DESIGN, SYNTHESIS AND PHARMACOLOGICAL EVALUATION OF SOME NEWER IMIDAZOLYL HETEROCYCLES AS POTENT *BTK* INHIBITORS FOR THE TREATMENT OF RHEUMATOID ARTHRITIS

A dissertation submitted to

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In partial fulfillment of the requirements for the award of the Degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL CHEMISTRY

Submitted by

A. MENAKA

REGISTRATION No.261515707

Under the Guidance of

Dr. R. PRIYADARSINI M. Pharm., Ph.D.,

Asst. Professor, Department of Pharmaceutical Chemistry

COLLEGE OF PHARMACY

MADRAS MEDICAL COLLEGE

CHENNAI-600 003

MAY-2017

COLLEGE OF PHARMACY
MADRAS MEDICAL COLLEGE
CHENNAI – 600003.
TAMIL NADU

CERTIFICATE

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Dr. A. JERAD SURESH, M. Pharm., Ph.D., M.B.A.,
Principal and HOD,
Department of Pharmaceutical Chemistry,
College of Pharmacy,
Madras Medical College,
Chennai- 03.

COLLEGE OF PHARMACY
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Dr. R.PRIYADARSINI, M. Pharm., Ph.D.,
Asst. Professor,
Department of Pharmaceutical Chemistry,
College of Pharmacy,
Madras Medical College,
Chennai- 03.

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A.Menaka

LIST OF ABBREVIATION

1.	RA	-	Rheumatoid Arthritis
2.	HLA	-	Human Leucocyte Antigen
3.	ACPs	-	Antigen Presenting Cells
4.	TNFα	-	Tumor necrosis Factor α
5.	IL	-	Interleukins
6.	AIDS	-	Acquired Immuno Deficiency Syndrome
7.	DMARDs	-	Disease Modifying Anti- Rheumatoid Arthritic Drugs
8.	NSAIDs	-	Non-Steroidal Anti-Inflammatory Drugs
9.	CADD	-	Computer Aided Drug Design
10.	CAMD	-	Computer Aided Molecular Drug designing
11.	CAMM	-	Computer Aided Molecular Modeling
12.	ADMET	-	Absorption, Distribution, Metabolism, Excretion and Toxicity
13.	IUPAC	-	International Union of Pure and Applied Chemistry
14.	GLIDE	-	Grid based Ligand Docking
15.	PDB	-	Protein Data Bank
16.	*BTK*	-	*Bruton's Tyrosine Kinase*
17.	PKC	-	Protein Kinase Enzyme
18.	QSAR	-	Quantitative Structural Activity Relationship
19.	FCgR	-	Fragment C gamma Receptor
20.	*MAOs*	-	*Mono Amino Oxidase*
21.	*JAK*	-	*Janus Kinase*
22.	*SYK*	-	*Spleen Tyrosine Kinase*

23. HBAL	-	Hydrogen Bond Acceptor Lipid
24. HBD	-	Hydrogen Bond Donor
25. HYP	-	Hydrophobic
26. TNCG	-	Trungated Newton Conjugate Gradient
27. OPLS	-	Optimized Potential for Liquid Simulation
28. RMSD	-	Root Meen Square Deviation
29. IR	-	Infra-Red Spectroscopy
30. NMR	-	Nuclear Magnetic Resonance
31. GC-MS	-	Gas Chromatography- Mass Spectroscopy
32. TMS	-	Tetra Methyl Silane
33. $CDCl_3$	-	Deuterated Chloroform
34. IAEC	-	Institutional Animal Ethics Committee
35. LD_{50}	-	Lethal Dose
36. IC_{50}	-	Inhibitory Concentration
37. CFA	-	Complete Freund’s Adjuvant
38. TLC	-	Total Differential Count
39. DLC	-	Differential Leucocyte Count

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I. INTRODUCTION

I.A. RHEUMATOID ARTHRITIS

A healthy immune system protects the body by attacking foreign bacteria and viruses, but an autoimmune disease causes the body to attack healthy tissue. Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease causing pain, swelling, stiffness and loss of function in the joints. In people with Rheumatoid Arthritis, white blood cells cause inflammation in the synovium. This causes the tissue that lines the walls of the joints to thicken and become swollen and painful when moved. The uncontrollable inflammation leads to joint erosion, a loss of motion and damage to many associated parts of the body such as lungs, heart[1].

a) CAUSES

Environmental factors, including smoking and pathogens have long been implicated as risk factors of rheumatoid arthritis [2, 3].

Genetic Factors

There are certain genes that may play a small role in the development of rheumatoid arthritis, although not directly. People who have one gene in particular, HLA (Human Leucocyte Antigen) could be 5 times more likely to get affected by rheumatoid arthritis. Research suggests these genes do not cause RA, but might make patients more vulnerable to developing it.

Environmental Factors

There are several environmental factors that, combined with a genetic predisposition, give people a greater risk to develop Rheumatoid Arthritis. These include:

- Bacteria and viruses
- Exposure to secondhand smoke
- Air pollution
- Insecticides

There are also several other risks that come from exposure to occupational hazards, such as mineral oils and Silica mineral (found in obsidian, granite, diorite, and sandstone)

Personal Factors

Gender: Both men and women are susceptible to rheumatoid arthritis, but the disease is far more common in women. In fact, 70 percent of people diagnosed with rheumatoid arthritis are women. This may be due to a variety of hormonal factors that are involved in the development of Rheumatoid Arthritis. Rheumatoid arthritis symptoms tend to improve or disappear completely during pregnancy, but frequent flares are common after the birth. Breastfeeding can also cause rheumatoid arthritis symptoms to flare.

Age: Rheumatoid Arthritis can affect a person at any age, but it typically presents in those between the ages of 40-60.

Family History: Those who have a family history of rheumatoid arthritis may have a higher chance of developing the disease.

b) SYMPTOMS

c) Common symptoms[4, 5] of rheumatoid arthritis are as follows

- Swollen joints
- Fatigue
- Significant weight loss
- Flulike symptoms and not feeling well
- Tender, warm, fever
- Pain and redness

d) PATHOPHYSIOLOGY

Rheumatoid Arthritis is an autoimmune disease, own body immune system that attacks the synovial tissue and other connective tissues. Once the initial immune response is triggered, cells of the immune system produce autoantibody and inflammatory cytokines, creating a cascade of inflammation. Chronic inflammations of synovial tissue lining of the joint capsule result in the proliferation of this tissue. The inflamed proliferating synovium in Rheumatoid Arthritis is called as pannus. This pannus invades the cartilage and bone surface, producing erosion of bone and cartilage leading to destruction of the joint[6].

The normal and Rheumatoid Arthritis joints were depicted in following figure[7].

Fig.No.1: Normal and Rheumatoid Arthritis Joints

Development of Arthritis in Rheumatoid Arthritis involved in the following steps[8]

Induction Phase

Induction phase involves release of antigen-presenting cells (APCs) and the citrulination of relevant proteins outside of joints along with monocyte or macrophage infiltration into the synovium, and local synovial cells. Fibroblasts and macrophages are activated leading to the secretion of proinflammatory cytokines of both the innate and adaptive immune systems.

Inflammation Phase

Self-antigens, notably citrullinated proteins, are presented in the context of Human leucocyte antigen Class II molecules that are characteristic of Rheumatoid Arthritis which leads to polyclonal activation of T-cells and B-cells and formation of germinal-like centers in the synovial tissue. This process is insufficiently controlled by regulatory T cells.

Self-perpetuation

Cartilage auto antigens (which are not normally accessible to the immune system) become exposed by damage and activates the immune system against cartilage tissue with further infiltration of pannus into the joints resulting in further destruction.

Destruction phase

Synovial fibroblasts and osteoclasts are activated by pro inflammatory cytokines such as TNF-α and IL-6. It may cause destruction of bone and cartilage

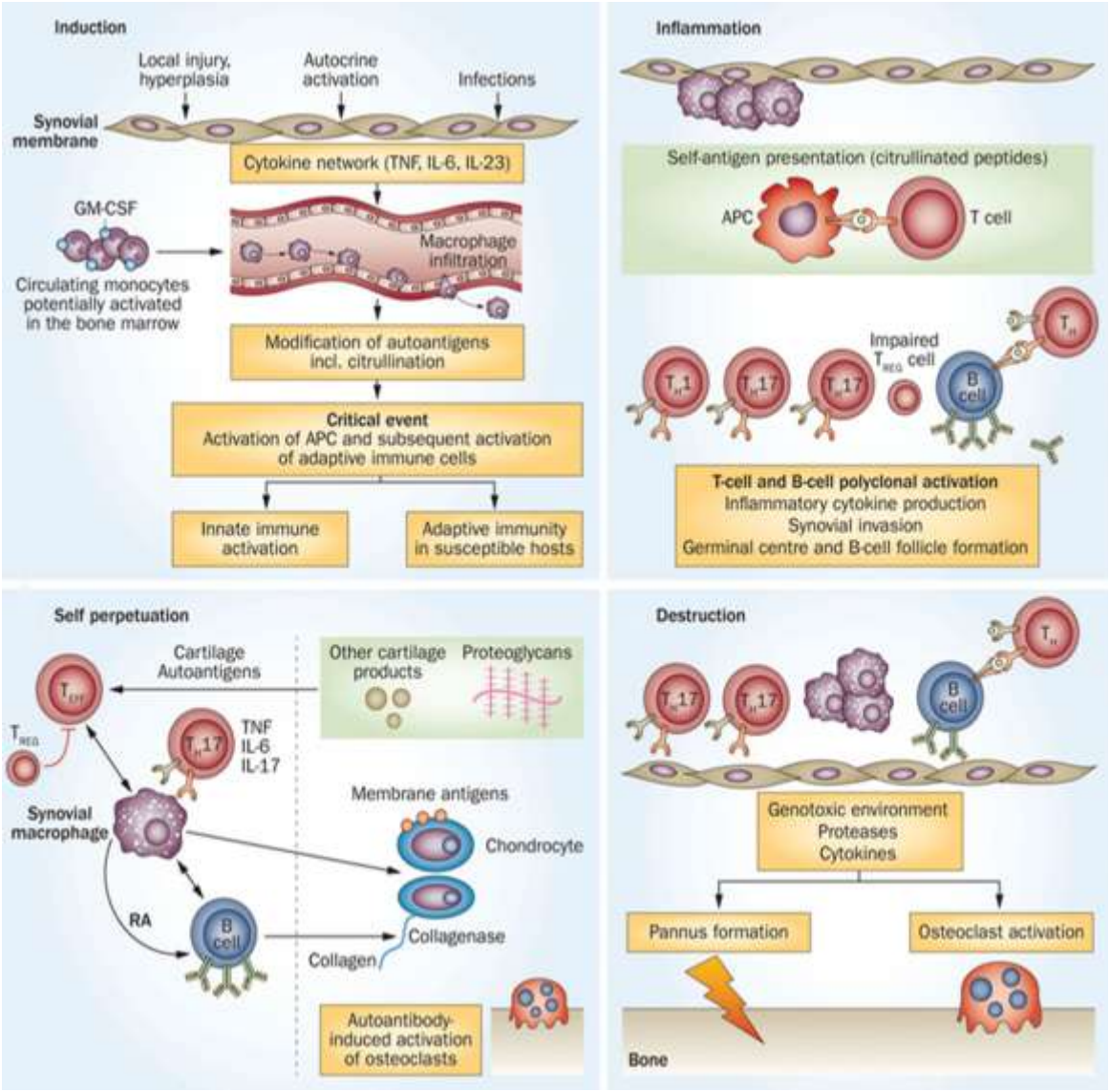


Fig.No.2: Stepwise development of arthritis in rheumatoid arthritis.

e) EPIDEMOLOGY

RA has probably received the greatest attention due to the progressive nature of its pathogenesis and its tremendous impact on public health and socioeconomics. RA affects 0.5-1% of the world population and in India about 15% people i.e. over 180 million people were affected by RA. This prevalence is higher than many well known diseases such as diabetes, AIDS and cancer[9].

f) TREATMENT

- **Therapeutic pyramid**

  Symptomatic drugs, shows a prompt action on pain and inflammation, but without any influence on disease progression.

- **Combination therapy** [10]

  Drugs that could modify the disease course and occasionally induce clinical remission - (DMARDs or disease modifying anti-rheumatic drugs) And Anti-Inflammatory agents such as steroids and Non-steroidal Anti-Inflammatory drugs (NSAIDs) are highly effective and should be part of an overall treatment program[5].

g) HISTORY OF ANTI-RHEUMATOID ARTHRITIC DRUGS

In olden days treatment for rheumatoid arthritis includes bloodletting, leeching, acupuncture, acupressure, moxibustion (use of heat), cupping etc. Heavy metals like gold, bismuth, arsenic and copper salts are also used in treatment of rheumatoid arthritis. Hippocrates, Galen used Willow extracts (containing salicin) to treat pain of rheumatoid arthritis and other forms of arthritis. Aspirin (1853), Salicylic acid (1929), Phenylbutazone (1949) and several other Non-steroidal anti-inflammatory agents also came into use. Payne in 1895 was the first to suggest the use of Quinine to treat RA. In 1957 Baguall used chloroquine and now hydroxychloroquine is still part of the Disease-modifying Anti-Rheumatic drugs (DMARDs). In 1949, Philip Hench first showed the successful use of cortisone in autoimmune diseases including rheumatoid arthritis. Methotrexate (1950)-folate antagonist also forms part of the DMARDs[11].

h) COMMONLY USED DRUGS

The commonly used drugs for the treatment of the Rheumatoid Arthritis are follows,

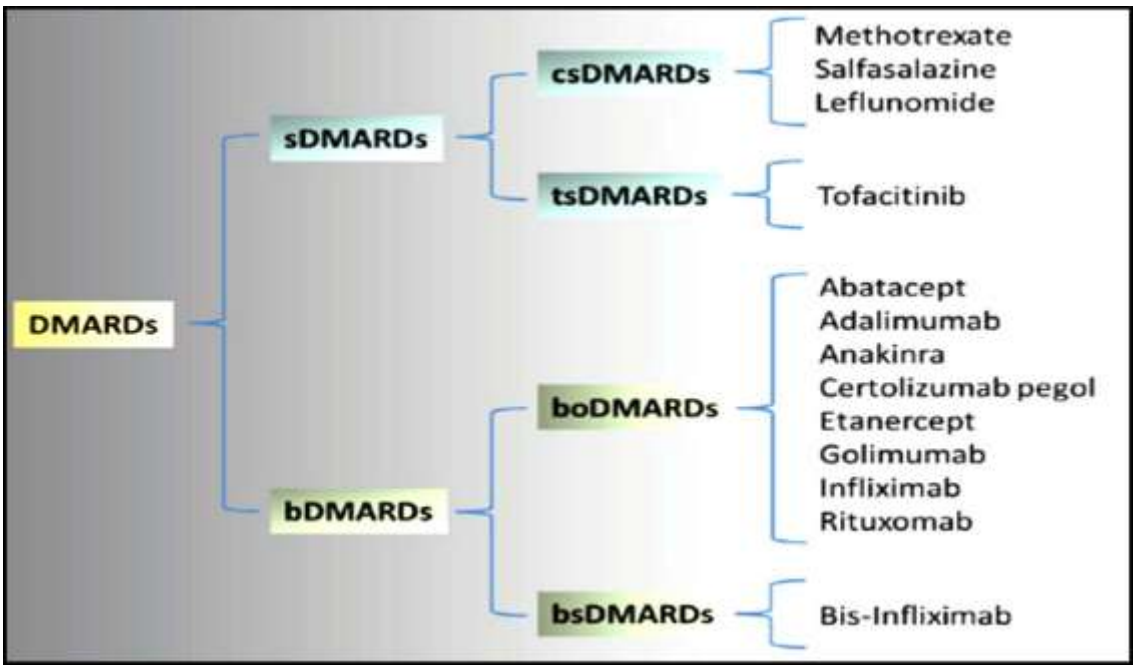
- **NSAIDs (Nonsteroidal anti-inflammatory drugs)**

  Ibuprofen, Naproxen sodium, Aspirin, Diclofenac sodium, Celecoxib, Nebumetone, Piroxicam, Indomethazine, Ketoprofen, Salsalate, Sulindac

- **Corticosteroids**

  Betamethasone, Prednisone, Dexamethasone, Cortisone, Hydrocortisone, Methylprednisolone, Prednisolone

- **Disease-modifying Anti-Rheumatic drugs (DMARDs)** [12]



i) NEED FOR NEW ANTI-RHEUMATOID ARTHRITIC DRUGS

Some of the reasons necessitating the emergence of newer anti- rheumatoid arthritic drugs are

- To improve the current course of treatment by shortening its total duration.
- To facilitate drug compliance by providing with less intensive supervision
- Drug with lesser frequency of administration which permits widely spaced intermittent treatment.
- To reduce the side effects which may include ringing in your ears, stomach irritation, heart problems and liver and kidney damage.
- To design a drug with higher efficacy and more specific in action by inhibiting the enzyme target which are responsible for inducing Rheumatoid Arthritis.

I. B. DRUG DESIGN

In the field of new discovery and development, Computational techniques are rapidly gaining popularity, implementation and appreciation Drug design is an inventive process of finding a new medication based on the knowledge of the biological target also known as rational drug design[13]. Drug design is frequently based on computer modeling techniques which are often referred to as Computer Aided Drug Design (CADD)

Different terms are being applied to this area, including computer aided drug design (CADD), computational drug design, computer aided molecular drug design (CAMD), computer aided molecular modeling (CAMM), rational drug design, Insilco drug design and computer aided rational drug design. Both computational and experimental techniques have complementary roles in drug discovery and development[14].

a) CADD ENTITIES

- Drug discovery and development process is streamlined by using the computing power.
- Chemical and biological information about ligands, targets in process of identification and optimization of new analogs.
- Design of Insilco filters to eliminate compounds with undesirable properties (ADMET) and select the most promising candidates.

Two types of drug design are as follows,

1. **Ligand Based drug design**

   Ligand based drug design (Indirect drug design) depends on knowledge of all other molecules which binds to the biological target.

2. **Structure based drug design**

   Structure based drug design (Direct drug design) mainly depends on knowledge of the three dimensional structure of the biological target[15].

b) PHARMACOPHORE MODELING

Pharmacophore modeling studies have become one of the major tool in the field of drug discovery. In 1909, Paul Ehrlich introduced the concept of Pharmacophore, who defined the pharmacophore as 'a molecular framework that carries (phoros) the essential features responsible for a drug's (Pharmacon) biological activity'.The IUPAC defines "A pharmacophore is ensemble of stearic and electronic features that is necessary to ensure the optimal supra-molecular interaction with a specific biological target and to trigger or block its biological response"[16].

Pharmacophore features

1. Hydrogen bond acceptor
2. Hydrogen bond donor
3. Hydrophobic
4. Hydrophobic aliphatic
5. Hydrophobic aromatic
6. Positive ionizable
7. Negative ionizable
8. Ring aromatoic

In order to identify novel ligands, the pharmacophoric features should match different chemical moieties with similar properties. A well-defined pharmacophore model includes both hydrophobic volumes and Hydrogen bond vectors.

Various ligand based and structure based methods involving pharmacophore modeling have been developed and extensively applied in the field of virtual screening, de nova design and lead optimization.

c) DOCKING STUDIES

Virtual screening techniques range from simple one up to sophisticated virtual docking methods aimed at fitting putative ligand molecules into the target receptor site[17]. In the field of drug design, docking predicts the preferred orientation of one molecule to the other when they bound to each other to form a stable complex[18]. Docking plays an important role in the molecular modeling as it is used to predict the binding orientation (affinity) of drug candidate to their protein targets[19].

1. Docking process

The docking process mainly involves the prediction of ligand conformation and orientation (posing) with active binding site of the target. Molecular docking process is compared to "lock-and-key" model. Here, the protein is considered as the "lock" and the ligand as a "key". The ligand and the protein adjust their conformation to achieve an overall "best-fit" which is referred as "induced-fit"[20, 21]

2. Docking methodology

Docking is an interactive procedure which generates the random ligand conformations for specified number of times, number of maximum trials[21].

Steps involved in docking

a. Protein preparation.
b. Selection of active site (Q-Site finder).
c. Ligand Preparation.
d. Docking Procedure.
e. Visualization / Interpretation of Docking.

a. Protein preparation

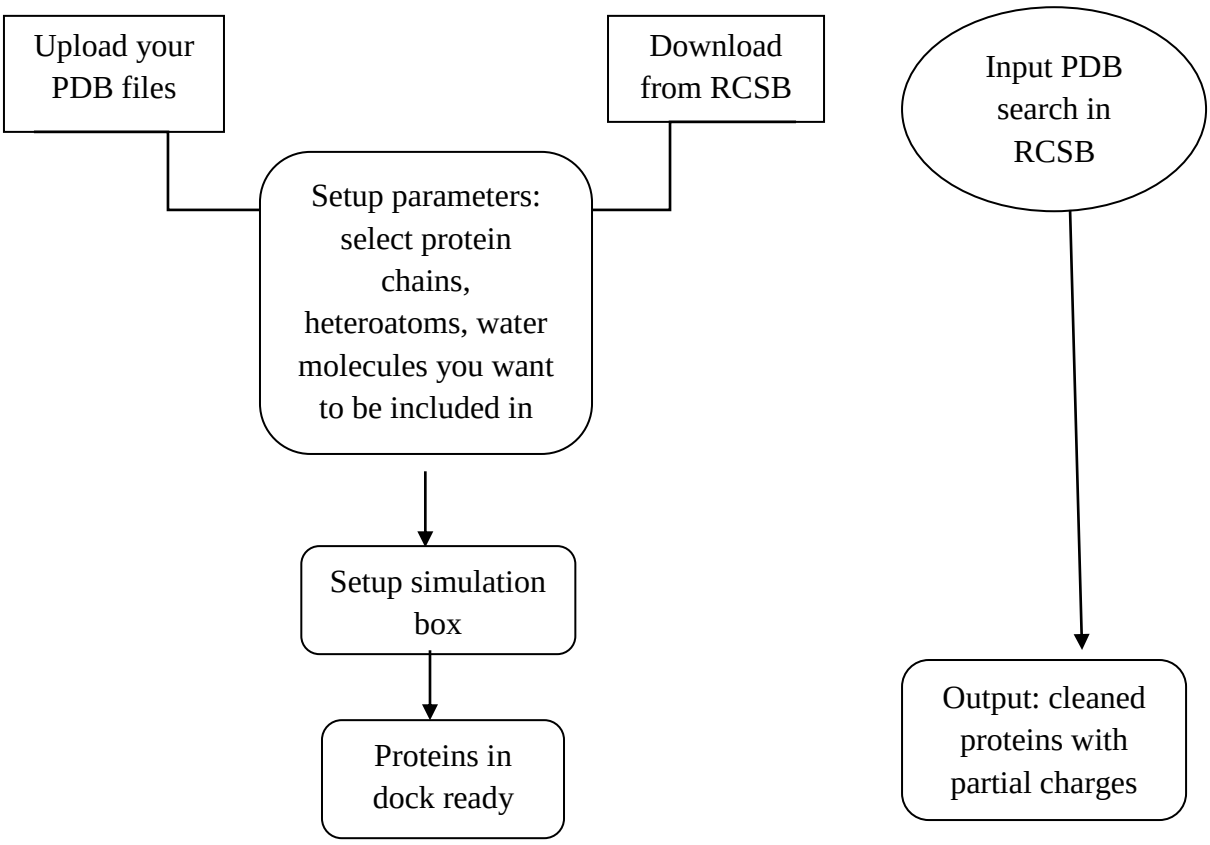


Fig.No.3: Preparation of proteins

b. Q-site finder

- Q-site finder an energy based method for protein ligand binding site prediction. During prediction we use the crystal structure of macromolecules (Receptor) with small substances (PDB ID: 5FBN)

c. Ligand preparation

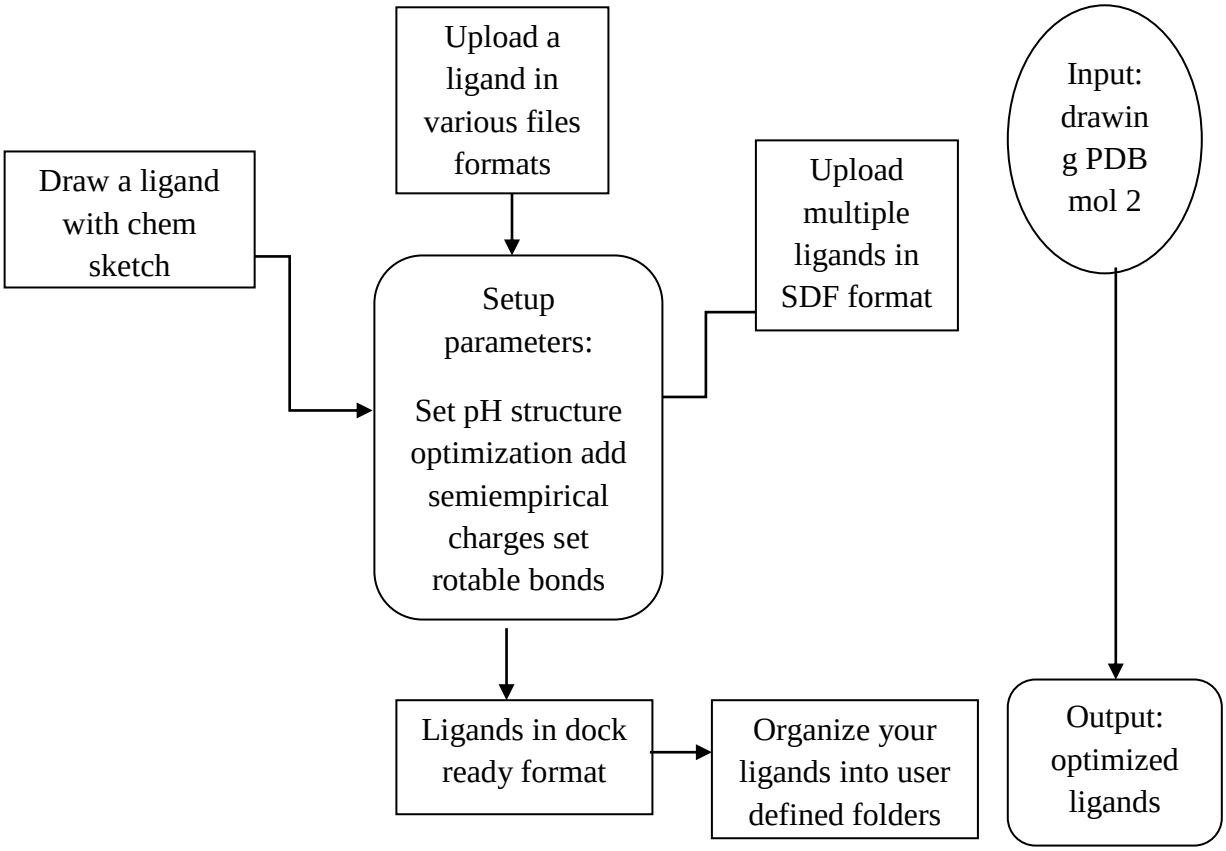


Fig.No.4: Preparation of ligands

d. **Docking procedure**

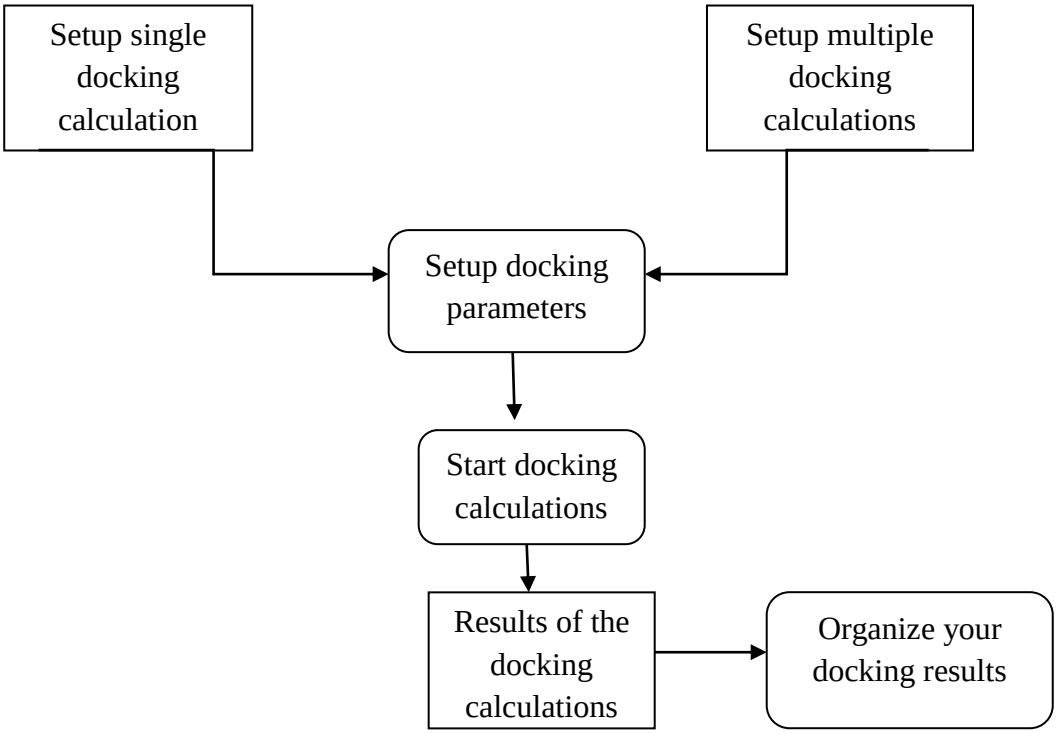


Fig.No.5: Docking calculations

e. **Visualization of docking results**

- Open molegro viewer
- File →Import molecule Select ligand map→Show interaction→Save image as ipeg format→Print screen→control+c→Open Paint→Paste→save

3. GLIDE 10.2

GLIDE 10.2 (Grid-Based Ligand Docking with Energetic) calculations are performed with impact version Maestro 10.2 (L. Schrodinger). Maestro input files of both ligands and the active site, including hydrogen atoms for the grid generation. The center of the grid enclosing box was defined by the center of the bound ligand as in the original PDB entry. The size of the bounding box for placing the ligand center was set to 12 A^o and a scaling factor of 0.9 was also applied to van der waals radii of ligand atoms for docking[22].

4. ARGUS LAB 4.1

Flexible docking simulation were performed using Argus 4.1 the binding region was defined using a grid 32 X 32 X 32 box centered on the centroid of the target. Default setting were used for all the remaining parameters. The top 50 poses were generated for each ligands. The docking posses were further energy minimized and Docking calculation by UFF method and the docking engine is Argus.

Applications

Docking is widely used in the field of molecular modeling and may be applied to,

- Hit identification- docking in combination with scoring function screens large databases of potential drugs Insilco to identify newer hits which binds to protein target.
- Lead optimization- used to predict the orientation and conformation of a ligand binds to a proteins[23-25].

I.C.TARGET ENZYME

BRUTON'S TYROSINE KINASE (BTK)

BTK is a member of the Tec family of cytoplasmic protein tyrosine kinase. It is predominantly expressed in hematopoietic cells, including B-cells, but also in cells of myeloid lineage such as macrophages, two cell lineages believed to be quite important in the treatment of inflammatory arthritis and other autoimmune diseases. *BTK* contains a pleckstrin homology (PH) domain, and Src homology SH3 and SH2 domains. *Btk* plays an important role in B cell development. Activation of B cells by various ligands is accompanied by *Btk* membrane translocation mediated by its PH domain binding to phosphatidylinositol-3,4,5-trisphosphate[26-28]. The membrane-located *Btk* is active and associated with transient phosphorylation of two tyrosine residues, Tyr551 and Tyr223. Tyr551 in the activation loop is transphosphorylated by the Src family tyrosine kinase, leading to autophosphorylation at Tyr223 within the SH3 domain, which is necessary for full activation[29,30]. The activation of *Btk* is negatively regulated by PKC through phosphorylation of *Btk* at Ser180, which results in reduced membrane recruitment, transphosphorylation and subsequent activation[31]. The PKC inhibitory signal is likely to be a key determinant of the B-cell receptor signaling threshold to maintain optimal *Btk* activity.

Mechanism of action

Animal models of arthritis suggest that *BTK* inhibition can result in inhibition of B-cell receptor-dependent cell proliferation and a reduction of inflammatory cytokine production from myeloid cells (including TNF, IL-1 and IL-6) by preventing signaling through the FCyR III receptor[32]. More recently, *BTK* has been shown to be a key element in the signaling pathways induced in macrophages by LPS stimulation of toll-like receptor 4 leading to the production of TNF-α, a key cytokine in RA pathogenesis.

Structure of *Bruton's Tyrosine Kinaase*[33]

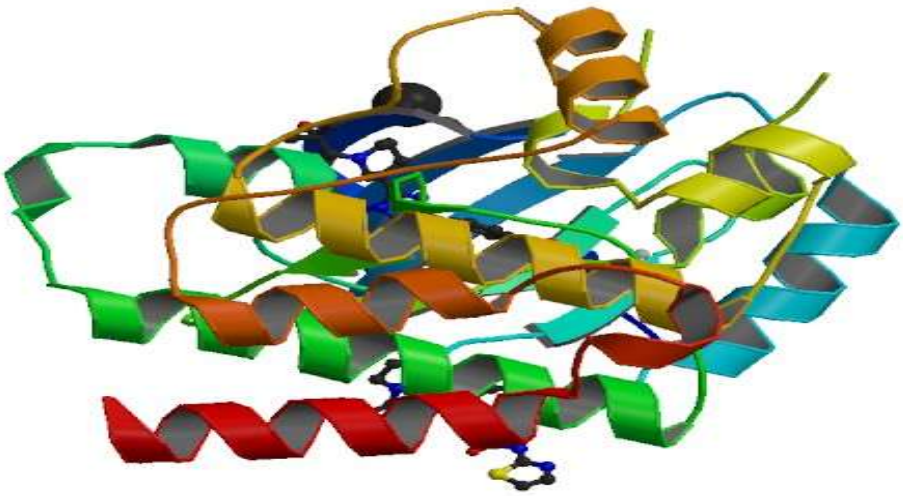


Fig.No.6: 3D Structure of BTK

Here in this study, we used computer aided drug design approaches to identify potent and novel inhibitors of *BTK* which has been considered as an attractive therapeutic target for B cell inhibition in the treatment of Rheumatoid Arthritis.

II. SYNTHETIC CHEMISTRY

The widespread occurrence of heterocyclic compounds in nature as alkaloids, vitamins, pigments in a variety of plants and animal cell constituents; their vital role in biological processes; their availability from agriculture wastes and their commercial value as solvents, dyes and pharmaceuticals are factors which have drawn the attention of organic chemists to this field.

IMIDAZOLE NUCLEUS

Imidazole is a planer five-member heterocyclic ring with 3C and 2N atom and in ring N is present in 1st and 3rd positions. Imidazole derivatives have occupied a unique place in the field of medicinal chemistry[34]. The high therapeutic properties of the imidazole related drugs have encouraged the medicinal chemists to synthesize a large number of novel chemotherapeutic agents. Numerous methods for the synthesis of imidazole and also their various structure reactions offer enormous scope in the field of medicinal chemistry[35].

STRUCTURE

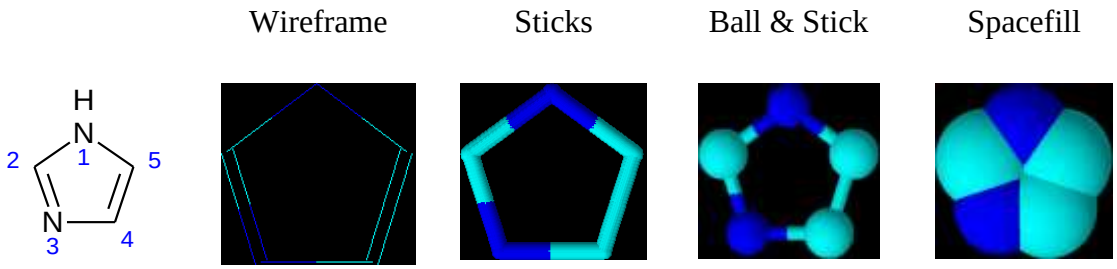


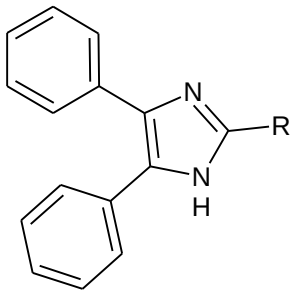
Fig.No.7: 2D & 3D Structure of Imidazole

PHYSIOCHEMICAL PROPERTIES

Imidazole are although weak bases, they are stronger bases than the isomeric pyrazoles. Like pyrazole, imidazole exhibits tautomerism as a result of which position 4 & 5 are equivalent[36, 37].

Nature	**:**	Solid
Molecular formula	**:**	$C_3 H_4 N_2$
Molecular weight	**:**	68.0772
Melting point	**:**	89 to 91 °C
Boiling point	**:**	256 °C
Solubility	**:**	Solubility in water
Density	**:**	1.23 g/ cm^3

SIGNIFICANCE OF SUBSITUTED IMIDAZOLES AS THERAPEUTIC AGENTS



2D structure of 4, 5 di phenyl 2-substituted imidazole

- It has been estimated that most of the therapeutic agents consists of heterocyclic compounds consisting of imidazole nucleus
- Heterocyclic ring like imidazole in many cases comprises the very core of the active moiety or Pharmacophore.
- During past decades, compounds bearing substituted imidazole have received much attention due to their chemotherapeutic value in the development of novel drugs
- Imidazole and 4,5 di phenyl 2-substituted imidazole has become an important part of many Pharmaceuticals.

MEDICINAL USES

Substituted imidazoles are widely employed as novel medicinal agents as they are found to possess the following biological activities

- Anti-tubercular activity.
- Anti-fungal and Anti-bacterial activity.
- Anti- inflammatory activity and analgesic activity.
- Anti- depressant activity
- Anti-cancer activity
- Anti-viral activity
- Anti-leishmanial activity

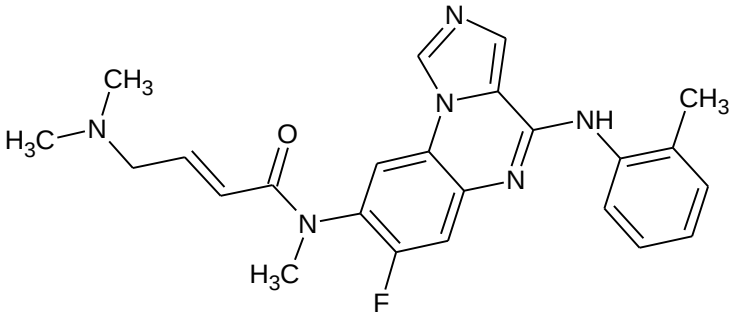
III. LITERATURE REVIEW

The review on following works provided, basic information about the target enzyme and designing of its inhibitors.

1. **Rohit Bavi *et.al*** [38] explored and reported some novel Inhibitors for *Bruton's Tyrosine Kinase* by 3D QSAR Modeling and Molecular Dynamics Simulation. Here in this study, 3D QSAR pharmacophore models were generated for *BTK* based on known IC_{50} values and experimental energy scores with extensive validation by subjecting into ADMET properties. Overall, this study suggest that the proposed ligands based on the generated Pharmacophore model may be more effective inhibitor for Rheumatoid Arthritis.

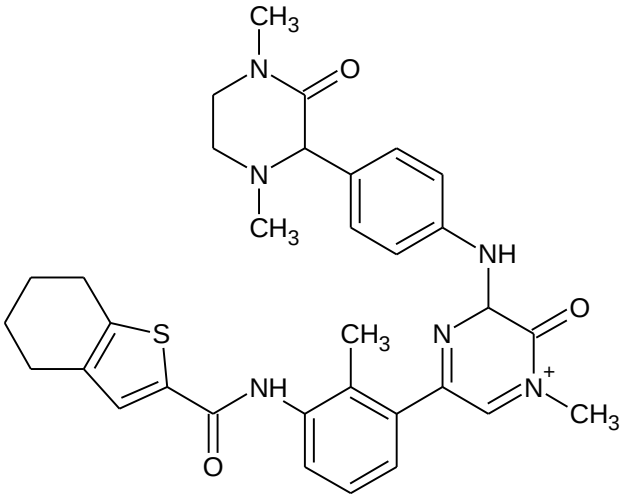
2. **Zhengying Pan D *et.al*** [39] developed a series of certain Selective Irreversible Inhibitors for Bruton's Tyrosine Kinase using a structural bioinformatics approach. Their capabilities to modulate *Btk's* activity were characterized both *In-vitro* and *In vivo*. Oral treatment with once-a-day dosing of compound 4 greatly inhibited disease development in a rodent rheumatoid arthritis (RA) model.

3. **Kyung-Hee Kim *et.al*[40]** synthesized and performed the SAR of a series of compounds are presented as well as the X-ray crystal structure of the lead compound **36** in complex with a gate-keeper variant of ITK enzyme. The lead compound showed good *In-vivo* efficacy in preclinical RA models.



lead compound 36

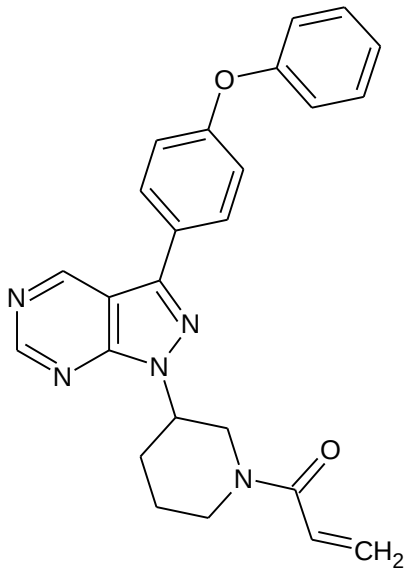
4. **Lichuan Liu *et.al*** [41] evaluated the anti-arthritic effect of GDC-0834 [*R-N*-(3-(6-(4-(1,4-dimethyl 3-oxopiperazin-2-yl) phenylamino)- 4-methyl-5-oxo-4,5-dihydropyrazin - 2-yl)- 2-methylphenyl)- 4,5,6,7-tetrahydrobenzo[*b*]thiophene-2-carboxamide], a potent and selective *BTK* inhibitor, and characterize the relationship between inhibition of *BTK* phosphorylation (*pBTK*) and efficacy. These findings suggest a high degree of *pBTK* inhibition is required for maximal activity of the pathway on inflammatory arthritis in rats.



Structure of GDC-0834

5. **Yan Lou *et.al*** [42] reported RN486, a Potent and Selective *Bruton's Tyrosine Kinase* as a potent inhibitor for the treatment of rheumatoid arthritis. Structure-based drug design was used to guide the optimization of a series of selective BTK inhibitors as potential treatments for Rheumatoid arthritis. introduction of a benzyl alcohol group and a fluorine substitution, each of which resulted in over 10-fold increase in activity.

6. **Akinleye *et.al*** [43] has reported Ibrutinib, as an novel *BTK* inhibitors in clinical development Recently *Bruton's tyrosine kinase* , a crucial terminal kinase enzyme in the B-cell antigen receptor (BCR) signaling pathway, has emerged as an attractive target for therapeutic intervention in human malignancies and autoimmune disorders. Ibrutinib, a novel first-in-human *BTK*-inhibitor, has demonstrated clinical effectiveness and tolerability in early clinical trials and has progressed into phase III trials.



Structure of Ibrutinib

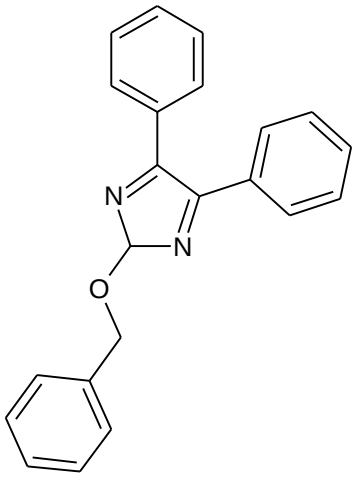
7. **Julie ADi Paolo *et.al*[44]** explained that *BTK* inhibition suppresses B-cell and myeloid cell–mediated arthritis. In macrophages, *BTK* inhibition abolishes FcgRIII-induced TNFa, IL-1b and IL-6 production. Accordingly, in myeloid- and FcgR-dependent autoantibody-induced arthritis, CGI1746 decreases cytokine levels within joints and ameliorates disease. These results provide new understanding of the function of *BTK* in both B- cell or myeloid cell–driven disease processes and provide a compelling rationale for targeting *BTK* in rheumatoid arthritis.

8. **Jian Liu *et.al* [45]** synthesized few 8-Amino-imidazo[1,5-*a*]pyrazines as Reversible BTK Inhibitors for the Treatment of Rheumatoid Arthritis. Selectivity is achieved through specific interactions of the ligand with the kinase hinge and driven by aminopyridine hydrogen bondings with Ser538 and Asp539, and by hydrophobic interaction of trifluoropyridine in the back pocket. These interactions are evident in the X-ray crystal structure of the lead compounds 1 and 3 in the complex with the BTK enzyme.

The review on following works provided ideas for synthesis of the Imidazole chemical entities as Anti-arthritic agents

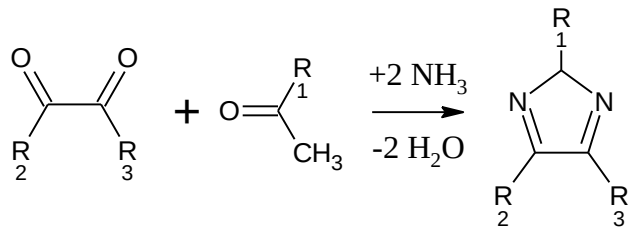
9. Anti -inflammatory and analgesic activity

Puratchikody A *et.al*[46] have synthesized certain 2-substituted-4, 5-diphenyl-1H-imidazoles and evaluated the anti-inflammatory activity based on Carrageenan-induced paw edema method. This 2-substituted-4, 5-diphenyl-1H-imidazoles compound shows maximum activity when and also compared with indomethacin which was used as reference drug.



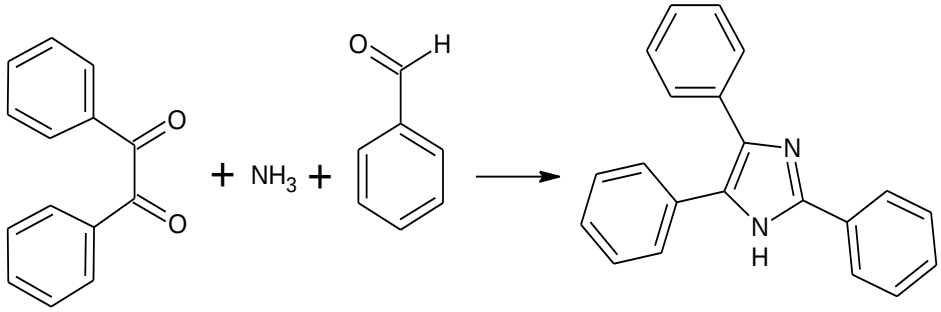
2-substituted-4, 5-diphenyl-1H-imidazoles

10. Debus *et.al* (1858)[47] synthesized some imidazolyl derivatives by using glyoxal and formaldehyde in ammonia. This synthesis, while producing relatively low yields, is still used for creating C substituted Imidazoles

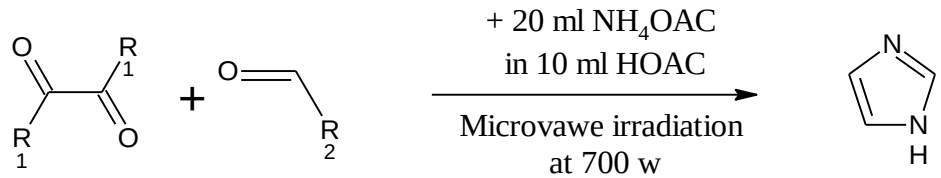


11. Radiszewski Synthesis

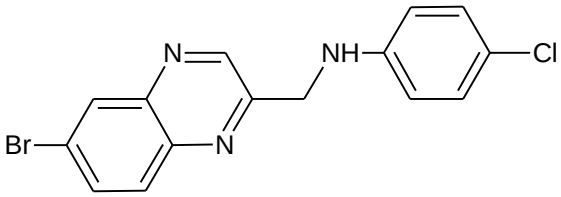
E.Lunt *et.al*[48] reported the condensation of a dicarbonyl compound, benzil and α-keto aldehyde or benzaldehyde in the presence of ammonia, to yield 2, 4, 5 triphenylimidazole.



12. Wahyuningrum *et.al* (2007)[49] synthesized certain 4,5-substituted imidazole derivatives utilizing microwave assisted organic synthesis (MAOS) method, by reacting with suitable diketone and some aldehyde or ketone, in order to investigate their corrosion inhibition mechanism on carbon steel surface.



13. Kavitha C.S *et.al*[50] has synthesized a series of 2-methylaminibenzimidazole derivatives and all the newly synthesized compounds were screened for analgesic and anti-inflammatory activities.



2-methylaminibenzimidazole derivatives

This compound shows analgesic activity and also compared with standard nimusulide drug.

The review on following works related to evaluation of anti-rheumatoid arthritic activity

16. **Caroline Charpin *et.al*** [51] identified new autoantibodies associated with Rheumatoid Arthritis disease duration less than one year. These autoantibodies could be used as diagnosis markers in RA patients.

17. **Shruthi SD *et.al*** [52] performed *In-vivo, In-vitro* and *in silico* anti-arthritic activity of a phytoconstituent, ellagic acid (EA) isolated from the leaves of *K. Reticulata* was isolated. The compound EA showed anti-arthritic activity which was found to be significant to that of the standard drugs aspirin and supports the traditional use of plant for treatment of rheumatism.

18. **A E van Ede *et.al*** [53] studied purine metabolism during treatment with methotrexate (MTX) in patients with rheumatoid arthritis (RA) and also discussed the relation of purine metabolism with efficacy and toxicity of MTX treatment.

19. **Lars Klareskog *et.al*** [54] explained adaptive immunity in rheumatoid arthritis: anticitrulline and other antibodies in the pathogenesis of rheumatoid arthritis. Several different triggering mechanisms are involved in forming an antibody repertoire that evolves before the onset of clinical disease, and where antibodies with different specificities may interact directly or indirectly with target organs in causing different arthritis-associated symptoms. The increasing understanding of the role of adaptive and specific immunity in RA creates opportunities for a new generation of interventions.

20. **Victoria Kell and Mark Genovese** [55] discovered a novel small molecule therapies which interrupt intracellular signalling through kinase inhibition. By interrupting one or more kinases it is possible to modulate the function of cellular structures such as surface receptors, signalling proteins and transcription of nuclear proteins and thus influence the behaviour of the cell types targeted. With these advances comes the significant potential to develop highly effective orally bioavailable therapeutics.

21. **Mohammed Munawar Hossain *et.al*** [56] performed an investigation of *in vitro* anti-arthritic and membrane stabilizing activity of ethanol extracts of three Bangladeshi plants. Inhibition and proteinase actions were evaluated to assess the anti-arthritic effect of the selected plant extracts. Membrane stabilizing activity of extract were assessed by using hypotonic solution and heat-induced method. In highest concentration of *Rhaphidophora glauca*, *Phrynium imbricatum*, *Steudnera colocasiifolia*, showed significant antiarthritic activity (53.16%, 69.62%, 62.03%) and membrane stabilizing activity (49.05%, 71.9%, 60.22%) compared with Diclofenac-Na.

22. **K. Sujatha *et.al*** [57] performed the assessment of *In-vitro* anti-arthritic activtiy of *Achyranthes Aspera linn.* The ethanolic extract of *Achyranthes aspera linn* was investigated for its anti- inflammatory activity by using protein inhibition assay method. The seven concentration of the extract and diclofenac sodium (10, 50,100, 200, 400,800, 1000 μg/ml) were used in this study as standard drug. The extract at the dose of 800 and 1000 μg /ml showed potent action on comparison with the standard diclofenac sodium.

IV. AIM AND OBJECTIVE OF WORK

AIM

The Main aim of this study is to identify, design, synthesize certain newer substituted imidazolyl derivatives as potent anti-rheumatoid arthritic agents with good predicted capability to inhibit the *BTK* involving Computational drug designing methods

OBJECTIVE OF WORK

The plan of work includes the following steps,

- Selection of target responsible for producing rheumatoid arthritis from the literature review which is carried out as part of the current research.
- Identification of common Pharmacophoric features responsible for inhibiting *Bruton's Tyrosine Kinase.*
- Designing a series of leads that selectively modulate the activities of *Bruton's Tyrosine Kinase* for exhibiting anti-rheumatoid arthritic activity.
- The binding mechanism of *BTK* receptor and newly designed leads have to be studied using molecular docking with *GLIDE 10.2* (PDB ID: 5fbn).
- Optimisation of designed leads based on the Lipinski rule of five, ADMET properties using *Molinspiration and Osiris software*
- Synthesis of certain optimized leads based on the synthetic feasibility.
- Characterization of chemical nature of the synthesized compounds by IR, ^{1}H NMR, ^{13}C NMR and GC-MS analysis.
- Docking studies of all the synthesized compounds using *GLIDE 10.2 and Argus Lab 4.0*
- Evaluation of all the synthesized compounds by performing *In-vitro* anti-arthritic activity -Protein inhibition assay method.
- *In-vivo* Pharmacological screening studies of the synthesized compounds
  - i) Evaluation of acute oral toxicity studies.
  - ii) Evaluation of Anti- Rheumatoid Arthritic activity.

V. EXPERIMENTAL WORK

Overall experimental flow chart is depicted below,

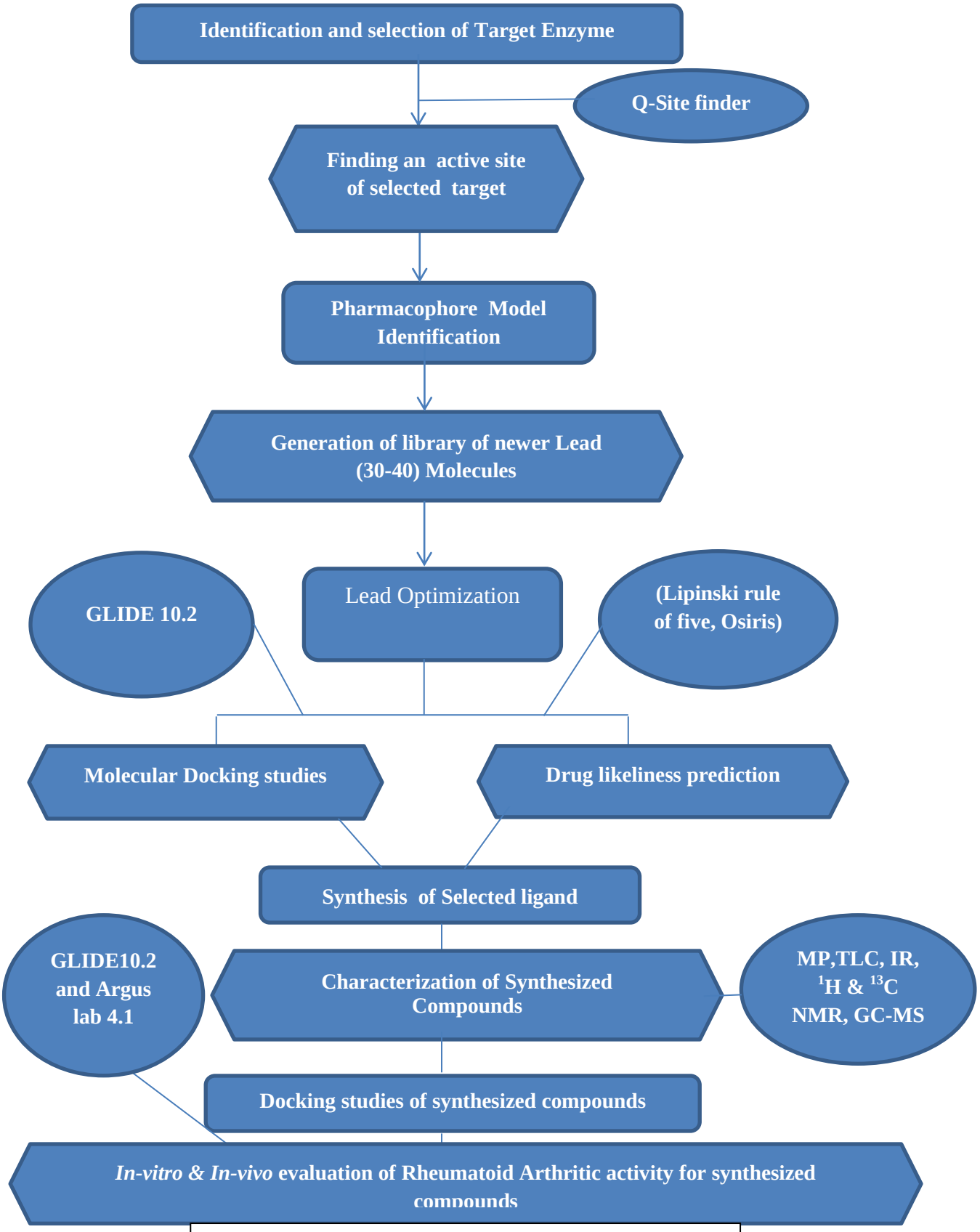


Fig No7: Flow chart

V.A.DRUG DESIGN

MATERIALS AND METHODS

1. SELECTION OF TARGET ENZYME

Protein Data Bank (PDB) is a crystallographic database for three dimensional structural data of large biological molecules such as proteins, Nucleic acid and Complex assemblies. The targets creating the greatest enthusiasm at this time for the treatment of Rheumatoid Arthritis and Inflammatory diseases include Janus-associated kinase (*JAK*), spleen tyrosine kinase (*SYK*), phosphodiesterase-4, *Bruton's tyrosine kinase* (*BTK*) and phosphatidylinositol-3 kinase. Ultimately human trials will help to understand the potential risks and benefits of these novel approaches across a number of diseases[58,59].

Using Q-site finder software tool, some of the recent and efficient PDB enzyme targets with low resolution were selected and further evaluated by its Resolution value, R Free, R value and optimised crystal ligand interaction details. Some of the selected receptors are listed below from which the highlighted best PDB target was used in this study

Table 1: List of target for Rheumatoid Arthritis

ABL	**ACK**	**CSK**	**FAK**	**FES**	**FRK**	**JAK**	**SRC-A**	**SRC-B**	**TEC**	**SYK**
ABL1	ACK1	CSK	FAK	FER	BRK	JAK1	FGR	BLK	BMX	SYK
ARG	TNK1	MATK	PYK2	FES	FRK	JAK2	FYN	HCK	***BTK***	ZAP 70
					SRMS	JAK3	SRC	LCK	ITK	
						TYK2	YES1	LYN	TEC	
									TXK	

Bruton's tyrosine kinase (BTK) is a cytoplasmic, non-receptor, tyrosine kinase which is expressed in most of the hematopoitic cells and plays an important role in the development, differentiation and proliferation of B-lineage cells, thus making *BTK* an efficient therapeutic target for the treatment of rheumatoid arthritis. Recent researchers suggested *BTK* as a therapeutic potential target to treat Rheumatoid Arthritis and Cancers which prompted us to select the *BTK* as the protein target in this study[60].

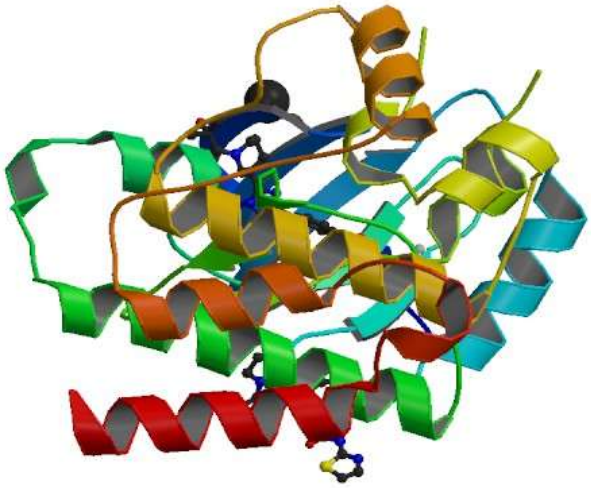


Fig.No.8: 3D Structure of BTK

Crystal Structure	:	*BTK*
PDB code	:	5FBN
Method	:	X-RAY DIFFRACTION
Resolution	:	1.8 Å
R-Value Free	:	0.226
R-Value Work	:	0.179

Active site of selected PDB

The active site of selected protein was identified by using **Q-site finder** software in which ligand mapping is used to show the active site of the existed co-crystal ligand .The active site of the selected target ezyme –*BTK* are identified as follows

5WE 702 (C) **:** Gln 459, Ser 453, Leu 460, Tyr 461, Asp 426, Gly 462, Ile 397, Trp 421, Tyr 425, Val 427.

5WF 701 (C) **:** Asn 484, Cys 481, Gly 409, Gly 480, Leu 408, **Thr 410**, Gly 411, Thr 476, Ala 478, Val 458, Glu 475, Met 477, Leu 528, Ala 428, Val 416, Leu 460, Met 450, **Asp 539**, Leu 542, Phe 442, **Phe 540**, Met 449, Val 463, Ile 472, Lys 430.

5WE 703 (D) **:** Gln 459, Ser 453, Trp 421, Leu 460, Ile 397, Ser 394, Gly 462, Val 427, Tyr 461, Met 450, Asp 426, Trp 421, Tyr 425.

5WE 704 (D) **:** Tyr 631, Arg 618, Glu 624.

5WE 705 (D) **:** Glu 624, Arg 618, Tyr 627, Thr 628, Tyr 631.

5WF 702 (D) **:** Asn 484, Cys 481, Gly 480, Leu 408, **Thr 410**, Thr 474, Gly 411, Leu 528, Glu 475, Val 416, Ala 428, **Asp 539**, Ala 446, Leu 542, Lys 430, Ser 538, Leu 460, Leu 542, Ser 538, Ala 446, Leu 542, Met 449, Phe 540, Val 463.

2. PHARMACOPHORE MODELING[61]

a) Pharmacophore identification

A Pharmacophore is defined as "a set of structural features in a molecule that is recognized at a receptor site and is responsible for that molecule's biological activity". When reviewing the efficient journals and research articles, (Rohit Bavi *et.al* reported the 3D QSAR Modeling of *Bruton's tyrosine kinase* in 2016) Pharmacophore model consisting of HBD, HBAL, HYP features was identified as the best model for designing *BTK* Inhibitors.

This pharmacophore model revealed that five chemical features consisting of one hydrogen bond acceptor lipid (HBAL), one hydrogen bond donor (HBD), three hydrophobic (HYP) features could effectively map all the chemical features. Hence, the above best hypothesis Hypo 1 was used as 3d structural query to screen the chemical databases for retrieving new potent *BTK* inhibitors.

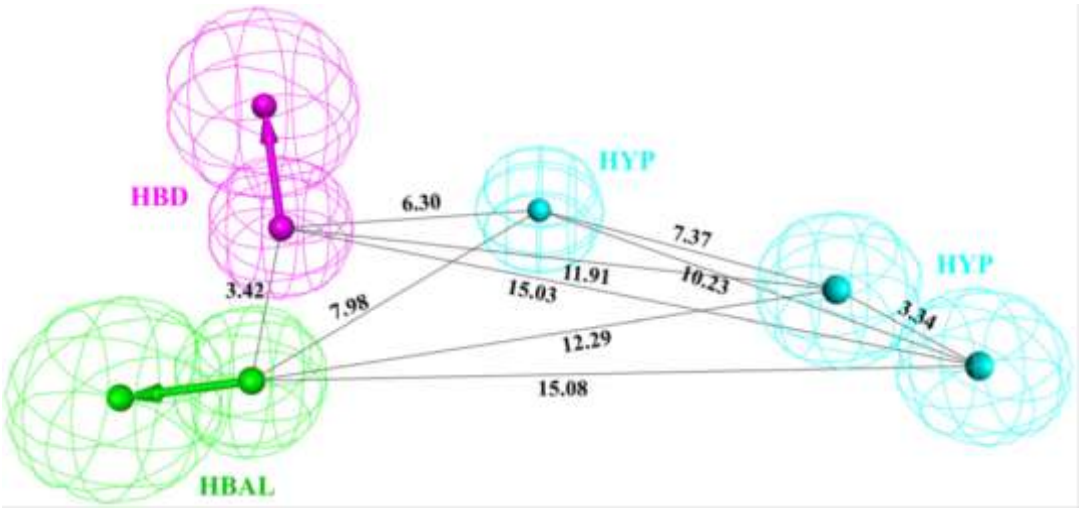


Fig.No.9: Chemical features of the best Pharmacophore 'Hypo 1' with its distance constraints.'Hypo 1' consists of one hydrogen bond acceptor lipid (HBAL: Green), one hydrogen bond donor (HBD: Magenta), three hydrophobic (HYP: Cyan) features.

Examples of HBAL and HBD Molecular Fragments

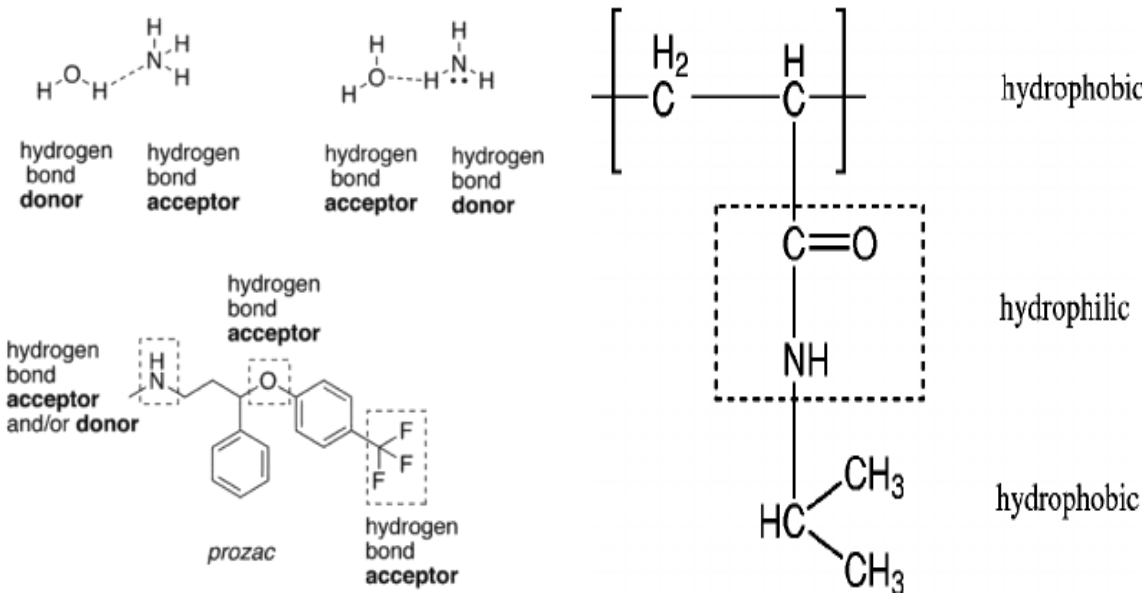


Fig.No.10: HBAL and HBD Molecular Fragments

b) Database screening

Scaffold hopping, or chemo type switching, is a technology that modifies the chemical scaffold of a bioactive compound retaining the activity and key interaction points, or the interacting molecular fragments of the parent compound.

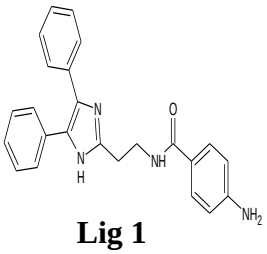
Based on the above quoted literature facts in designing potent *BTK* inhibitors, the target screening library was designed by using molecular fragments from a relatively narrow and low molecular weight range (350-5000D), selected diversity at both the putative "scaffold" core. The analogue library was generated by modifying the respective functional groups with sterically and conformationally allowed substituents using the reagent database and a combinatorial design model.

Table.No.2: Molecular fragments used in construction of library

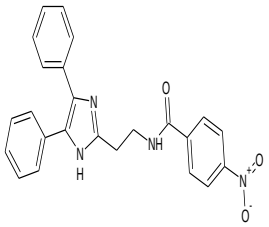
HBD	HBA	Linker	Hydrophobic tail
Imidazole, Benzimidazole, Aminothiazole, Phenolic-OH, Aniline, Alkyl amines, Hydrazines, Morpholine	C=O of aliphatic and aromatic amides , C=O of aromatic ketones,	Phenyl, Methyl, Ethyl, Phenoxy,	Phenyl, Diazole, Pyridine Triazole, Quinaxoline, dimethyl benzene,

Construction of a Large Virtual Scaffold Library

A library consisting of nearly new 30-40 lead molecules as potent *BTK* inhibitors was generated based on the knowledge of binding interaction of Ligand with the protein and also the common features necessary for the biological activity of molecule .The Hypo1 model (one hydrogen bond acceptor lipid (HBAL), one hydrogen bond donor (HBD), three hydrophobic (HYP) features) was used to screen knowledge database and a virtual scaffold library consisting of newly designed 33 molecules has been constructed have been shown in below,



Lig 1



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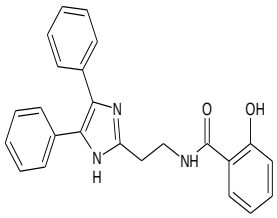

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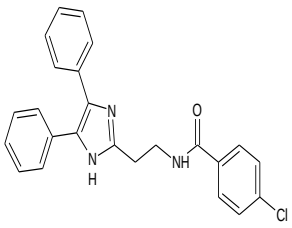

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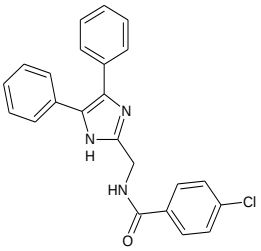

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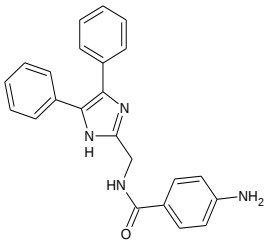

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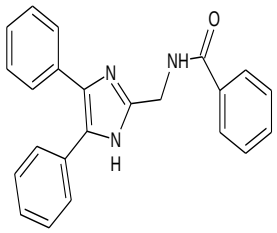

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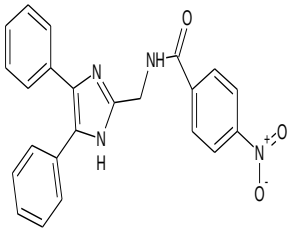

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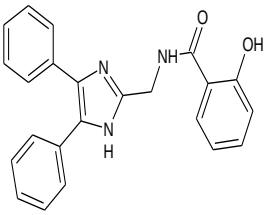

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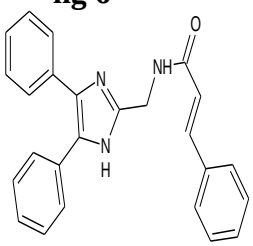

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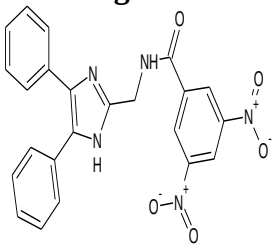

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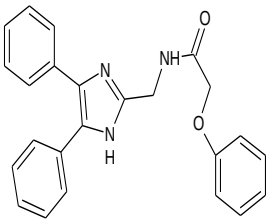

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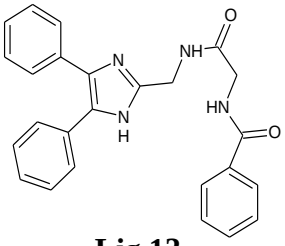

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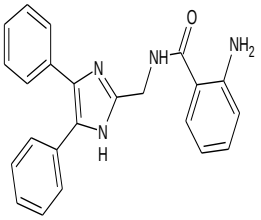
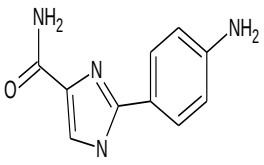


lig 14



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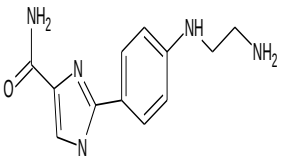
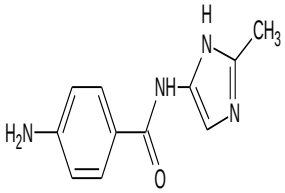
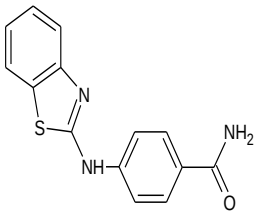


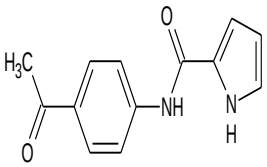
lig 16



Lig 17



lig 18



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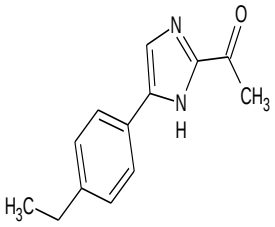
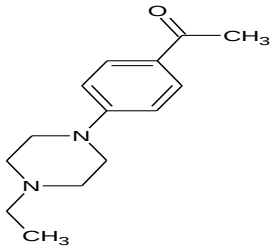


lig 20



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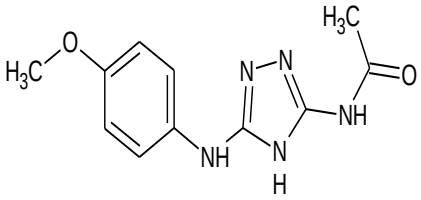
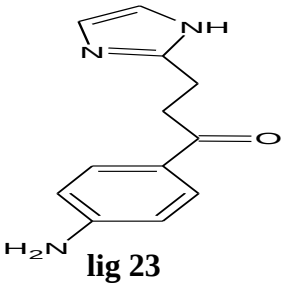
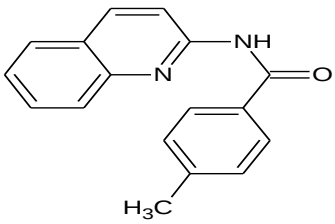


lig 22



lig 23



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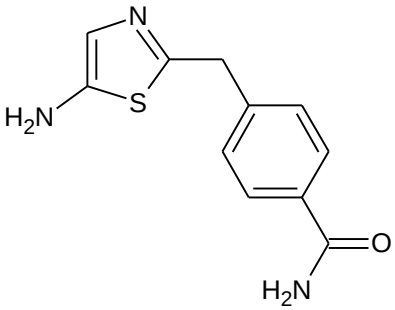

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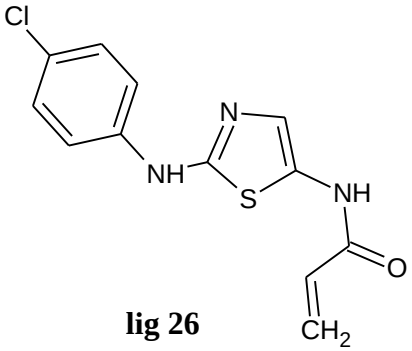
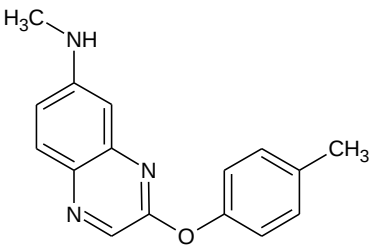
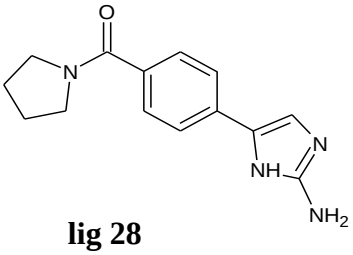


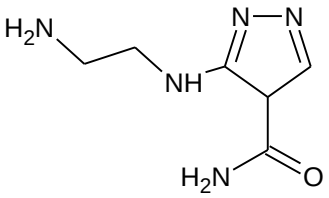
lig 26



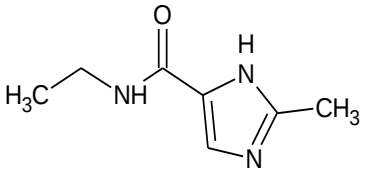
Lig 27



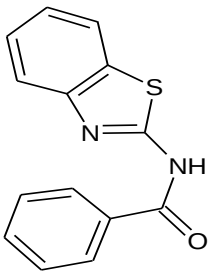
lig 28



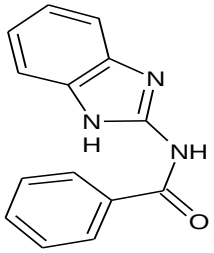
lig 29



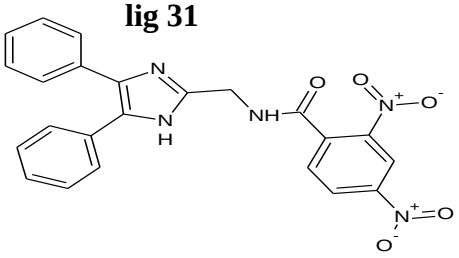
Lig 30



lig 31



lig32



lig 33

Fig.No.11: Virtual scaffold library of newly designed leads as BTK inhibitors

3. LEAD OPTIMIZATION

All the designed ligands (1-33) were optimized by subjecting to Docking studies, ADMET properties, Lipinski's rule of five, Novelty prediction and Toxicity prediction to refine further.

A. DOCKING STUDIES OF DESIGNED LEADS

a) Preparation of protein target structure

The crystal structure of *BTK* inhibitor (PBD ID: 5FBN, Resolution 1.8 Å) was downloaded from the Protein Data Bank (www.rcsb.org) and employed for Glide 10.2 docking studies. For GLIDE (Schrodinger) calculations, *BTK Target enzyme* was imported to Maestro (Schrodinger), the co-crystalized ligands were identified and removed from the structure and the protein was minimized using the protein preparation wizard (shipping by Schrodinger) by applying an OPLS force field. The refinement procedure is recommended by Schrodinger because Glide uses the full OPLS force field at an intermediate docking stage and is claimed to be more sensitive to geometrical details than other docking tools. Water molecule were removed and the hydrogen atom were added to the structure. The most likely position of hydroxyl and H atoms were added to the structure. Minimization were performed until the average root mean square deviation of the Non hydrogen atoms reached 0.3 Å.

b) Ligand preparation

3D structure of *BTK* ligands were built and adjusted for bond order using *Maestro 10.2* (Schrodinder suite). Geometry minimization were performed on all ligand using the OPLS force field and the Truncated Newton Conjugate Gradient (TNCG) minimization algoritham. Optimization of ligand conformations were converged to a gradient RMSD below 0.05 KJ/Mol or continued to a maximum of 1000 interaction, at which point there were negligible changes in RMSD gradients.

The structures supplies to docking tool must meet the following condition,

1. They must be three- dimentional (3D).
2. They must have realistic bond lengths and bond angles.
3. They must each consist of a single molecule that has no covalent bonds to the receptor, with no accompanying fragments, such as counter ions and solvent molecules.
4. They must have all their hydrogens (field valences).
5. They must have an appropriate protonation state for physiological pH value around 7.

c) Docking studies using GLIDE 10.2

Docking was performed on *BTK*, twenty distinct poses of each ligand in the active site of *BTK* were generated. The binding pocket of *BTK* is made up of key residues **Thr 410, Asp 539**, **Phe 540.** The ligand IPAA, ISA, IPABA, IHA and IAA were found to have very high fitness score. It was also observed that the hydrogen bond and pi-pi static interactions between the target and the ligands.

B. DRUG LIKENESS SCREENING

The properties which can differentiate drugs other chemicals can be considered as drug like properties. Drug likeness is qualitative concept used in drug design for how druglike a substances is to be an effective drug. Drug likeliness properties was performed for all the newly designed *BTK* inhibitors by using different online softwares like *Lipinski's rule of five, Osiris online software, Molinspiration.*.

a) *Lipinski's rule of five*

Lipinski's rule of five is a rule of thumb to evaluate drug likeness, or to determine if a chemical compound with a certain pharmacological or biological activity has properties that would make it a likely orally active drug in humans. The rule was formulated by Christopher A. Lipinski in 1997, based on the observation that most medication drugs are relatively small and lipophilic molecules.

The modification of the molecular structure often leads to drugs with higher molecular weight, more rings, more rotatable bonds and higher lipophilicity. The rule is

called “Rule of 5”, because the border values are 5, 500, 2*5. The rule of five was calculated using a software tool called *Molinspiration* online database.

Lipinski’s rule says that, in general, an orally active drug has no more than one violation of the following criteria,

- Log P value should be less than 5
- Hydrogen bond donor less than 5
- Hydrogen bond acceptor less than 10
- Molecular weight under 500 Daltons and
- Not more than 10 rotatable bonds.

b) ADMET properties

The ADMET provides components that calculate predicted absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties for ligand. A drug substances must be characterized by optimal solubility to both water and fat. The model compound for cellular membrane is octanol, so the logarithm of the octanol/water partition co efficient, known as log $P_{o/w,}$ is used to estimate the solubility. Solubility in water can be estimated from the number of hydrogen bond donors vs the alkyl side chains in the molecule. Too many hydrogen bond donors, on the other hand, leads to low fat solubility, so the drug cannot penetrate the cell wall.

During ADMET investigation the ligands were checked for low blood brain barrier (BBB), optimal solubility, good absorption. The toxicological properties like Teratogenicity, Mutagenicity, Irritant effect and Reproductive effect of designed ligands were predicted using *Osiris* online software. Prediction results are evaluated and color coded. Unfavorable properties or those with high risk of side effect like mutagenicity or poor intestinal absorption in Red, Green whose indicate Drug-conform.

RESULTS AND DISSCUSSIONS

A. DOCKING STUDIES

Docking studies was performed on all the newly designed 33 *BTK* inhibitors retrieved from virtual screening using *Glide 10.2*. Results of predicted activity of all the designed ligands with fitness score are tabulated below

Table.No.3: List of docking score for designed ligands

S. No	Ligand	Docking score Using Glide 10.2
1	Lig 1	-7.864
2	Lig 2	-9.264
3	Lig 3	-7.159
4	Lig 4	-8.141
5	Lig 5	-7.426
6	Lig 6	-6.808
7	Lig 7	-7.050
8	Lig 8	-7.567
9	Lig 9	-6.437
10	Lig 10	-9.684
11	Lig 11	-7.267
12	Lig 12	-7.970
13	Lig 13	-7.770
14	Lig 14	-7.632
15	Lig 15	-6.460
16	Lig 16	-6.800
17	Lig 17	-4.731
18	Lig 18	-6.351
19	Lig 19	-5.651
20	Lig 20	-5.987

S. No	Ligand	Docking score Using Glide 10.2
21	Lig 21	-6.262
22	Lig 22	-4.279
23	Lig 23	-5.887
24	Lig 24	-7.828
25	Lig 25	-7.229
26	Lig 26	-4.807
27	Lig 27	-6.134
28	Lig 28	-5.670
29	Lig 29	-5.000
30	Lig 30	-5.034
31	Lig 31	-6.422
32	Lig 32	-7.891
33	Lig 33	-6.943

B. DRUG LIKELINESS PROPERTIES

When all the 33 newly designed ligands were subjected to drug likeness prediction studies all were found to exhibit the drug likeness properties. But based on the synthetic feasibility only Ligand (6), Ligand (9), Ligand (14), Ligand (13), Ligand(12) were selected as lead for synthesis as potent *BTK* inhibitor and hence the results of selected ligands alone were discussed below,

a) ***Lipinski's rule of five***

The Lipinski's rule of five was performed by using *Lipinski's rule of five* molecular properties calculator online software. All the selected ligands Ligand 6, Ligand 9, Ligand 14, Ligand 13, Ligand 12 were found to pass the Lipinski rule of five and the results were tabulated below,

Table.No.4: Lipinski rule of five reports for the selected ligands

S. No	Ligands	Molecular Mass (Dalton)	logP(<5)	Hydrogen bond donor(<5)	Hydrogen bond acceptor(<10)	Molar Refractivity(40-130)
1	**Ligand 6**	364.00	3.239840	3	4	108.616570
2	**Ligand 9**	365.00	3.362879	2	4	105.868965
3	**Ligand 14**	379.00	3.422479	1	4	110.375664
4	**Ligand 13**	404.00	3.254679	1	5	117.703156
5	**Ligand 12**	364.00	3.239840	3	4	108.616570

Table.No.5: Molecular properties of selected ligands

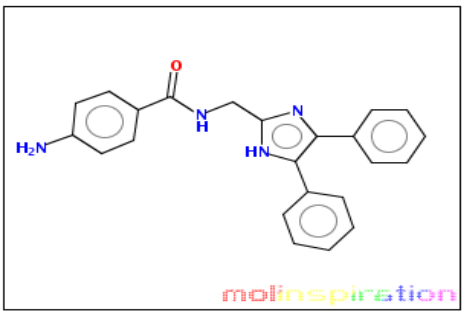
S. No	Ligands	No of atoms	No of rotatable bonds	TPSA	Violation
1	Ligand 6	28	5	83.80	0
2	Ligand 9	28	5	78.01	0
3	Ligand 14	29	**7**	67.02	0
4	Ligand 13	28	5	83.80	0
5	Ligand 12	31	7	86.88	0

b) ADMET properties

The ADMET results of the selected ligands were depicted in the following images,

Ligand 6

Biological activity



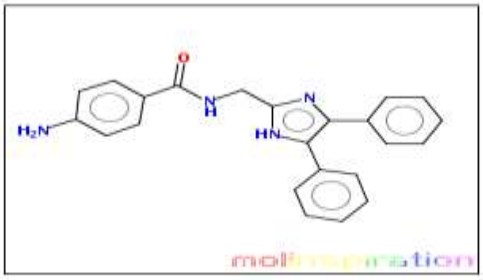
Molinspiration bioactivity score v2016.03

GPCR ligand	0.22
Ion channel modulator	0.11
Kinase inhibitor	0.44
Nuclear receptor ligand	-0.26
Protease inhibitor	0.06
Enzyme inhibitor	0.28

Get data as text (for copy / paste).

Get 3D geometry BETA

Physiochemical Properties



Molinspiration property engine v2016.10

miLogP	3.04
TPSA	83.80
natoms	28
MW	368.44
nON	5
nOHNH	4
nviolations	0
nrotb	5
volume	338.57

Get data as text (for copy / paste).

Get 3D geometry BETA

Toxicity profile

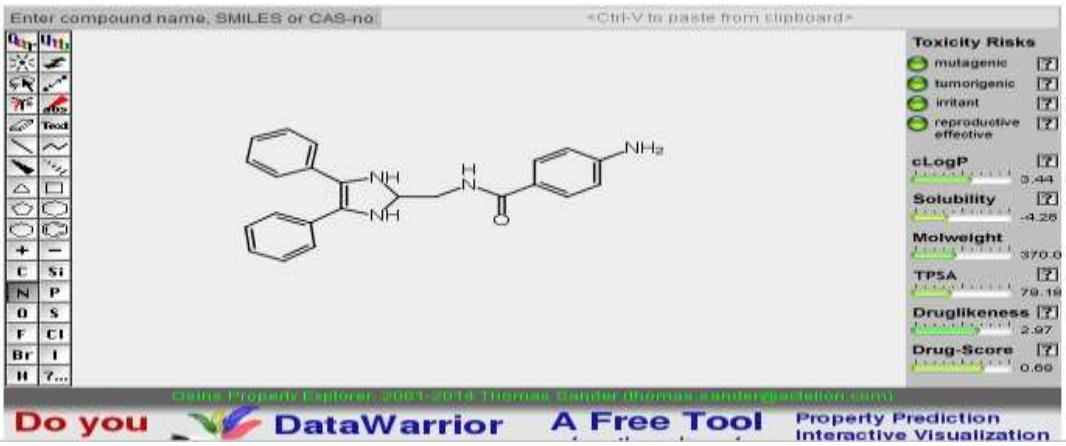
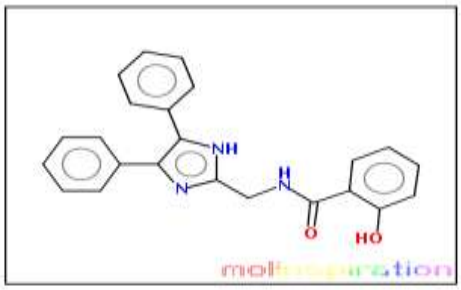


Fig.No.12: Biological activity, Physiochemical Properties and Toxicity profile images for Ligand 6

Ligand 9

Biological activity



Molinspiration property engine v2016.10

miLogP	4.46
TPSA	78.01
natoms	28
MW	369.42
nON	5
nOHNH	3
nviolations	0
nrotb	5
volume	335.30

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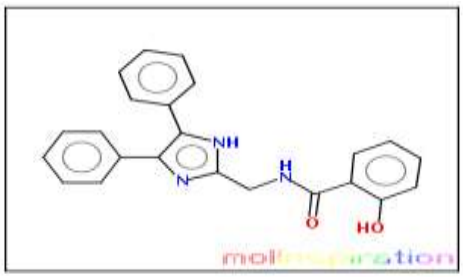
Get 3D geometry BETA

Physiochemical Properties



Molinspiration property engine v2016.10

miLogP	4.46
TPSA	78.01
natoms	28
MW	369.42
nON	5
nOHNH	3
nviolations	0
nrotb	5
volume	335.30

Get data as text (for copy / paste).

Get 3D geometry BETA

Toxicity profile

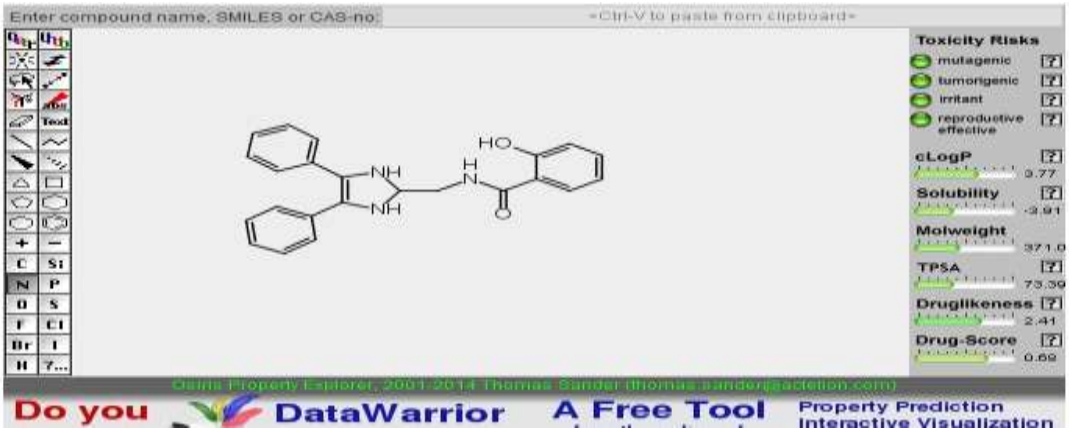
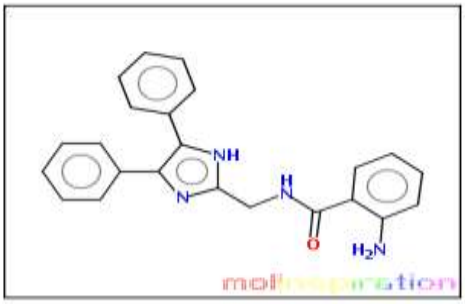


Fig.No.13: Biological activity, Physiochemical Properties and Toxicity profile images for Ligand 9

Ligand 14

Biological activity



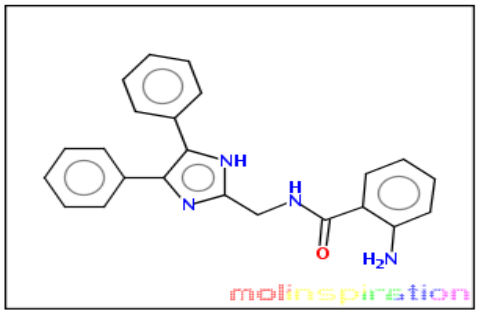
Molinspiration bioactivity score v2016.03

GPCR ligand	0.20
Ion channel modulator	0.10
Kinase inhibitor	0.43
Nuclear receptor ligand	-0.28
Protease inhibitor	0.04
Enzyme inhibitor	0.27

Get data as text (for copy / paste).

Get 3D geometry BETA

Physiochemical Properties



Molinspiration property engine v2016.10

miLogP	3.40
TPSA	83.80
natoms	28
MW	368.44
nON	5
nOHNH	4
nviolations	0
nrotb	5
volume	338.57

Get data as text (for copy / paste).

Toxicity profile

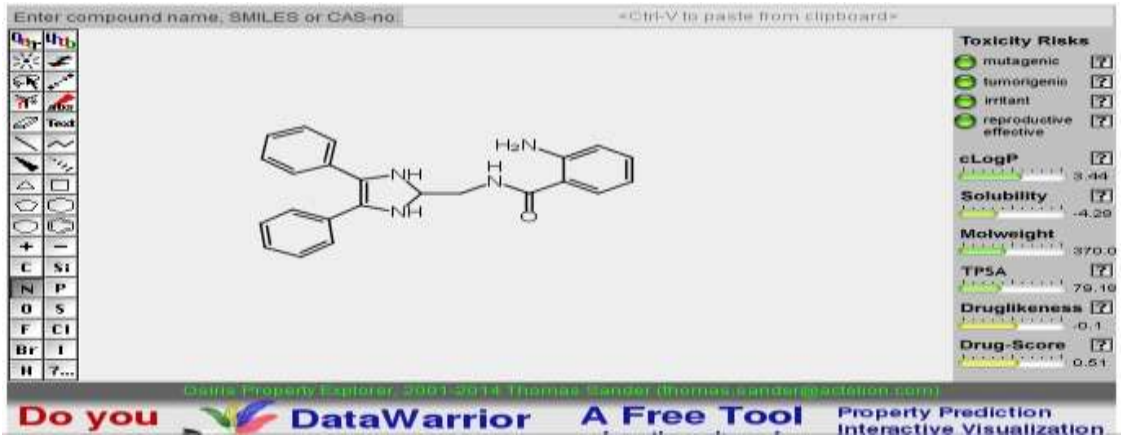
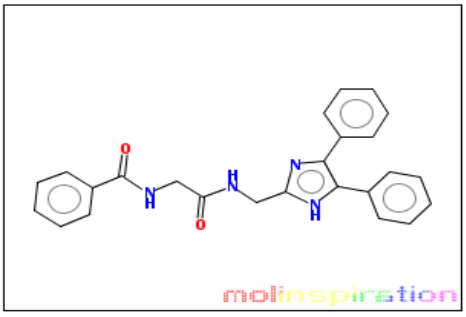


Fig.No.14: Biological activity, Physiochemical Properties and Toxicity profile images for Ligand 11

Ligand 13

Biological activity



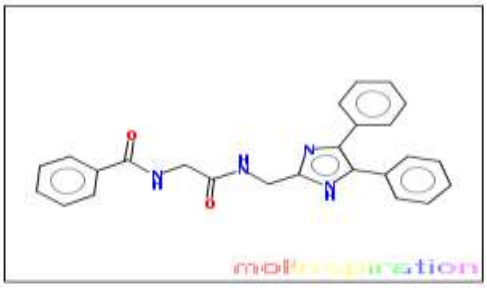
Molinspiration bioactivity score v2016.03

GPCR ligand	0.20
Ion channel modulator	-0.01
Kinase inhibitor	0.23
Nuclear receptor ligand	-0.32
Protease inhibitor	0.06
Enzyme inhibitor	0.15

Get data as text (for copy / paste).

Get 3D geometry BETA

Physiochemical Properties



Molinspiration property engine v2016.10

miLogP	3.58
TPSA	86.88
natoms	31
MW	410.48
nON	6
nOHNH	3
nviolations	0
nrotb	7
volume	375.47

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Get 3D geometry BETA

Toxicity profile

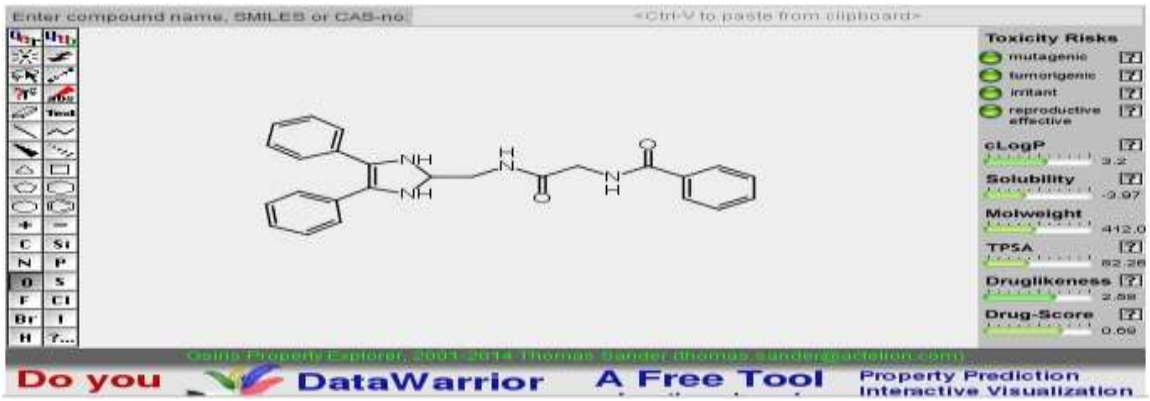
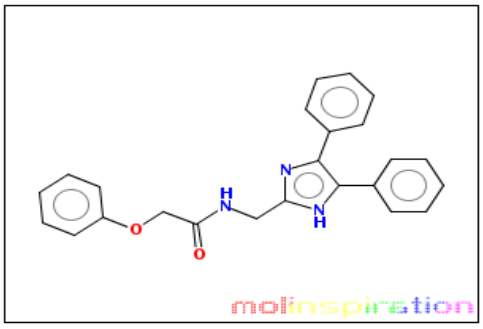


Fig.No.15: Biological activity, Physiochemical Properties and Toxicity profile images for Ligand 13

Ligand 12

Biological activity



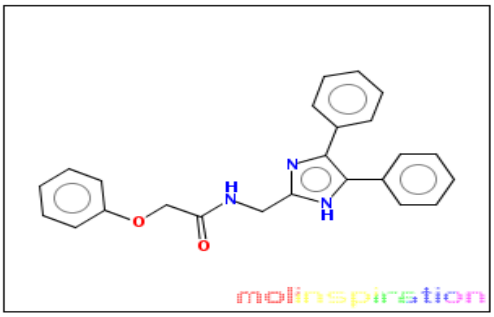
Molinspiration bioactivity score v2016.03

GPCR ligand	0.12
Ion channel modulator	−0.06
Kinase inhibitor	0.18
Nuclear receptor ligand	−0.32
Protease inhibitor	−0.04
Enzyme inhibitor	0.09

Get data as text (for copy / paste).

Get 3D geometry BETA

Physiochemical Properties



Molinspiration property engine v2016.10

miLogP	3.94
TPSA	67.02
natoms	29
MW	383.45
nON	5
nOHNH	2
nviolations	0
nrotb	7
volume	353.07

Get data as text (for copy / paste).

Get 3D geometry BETA

Toxicity profile

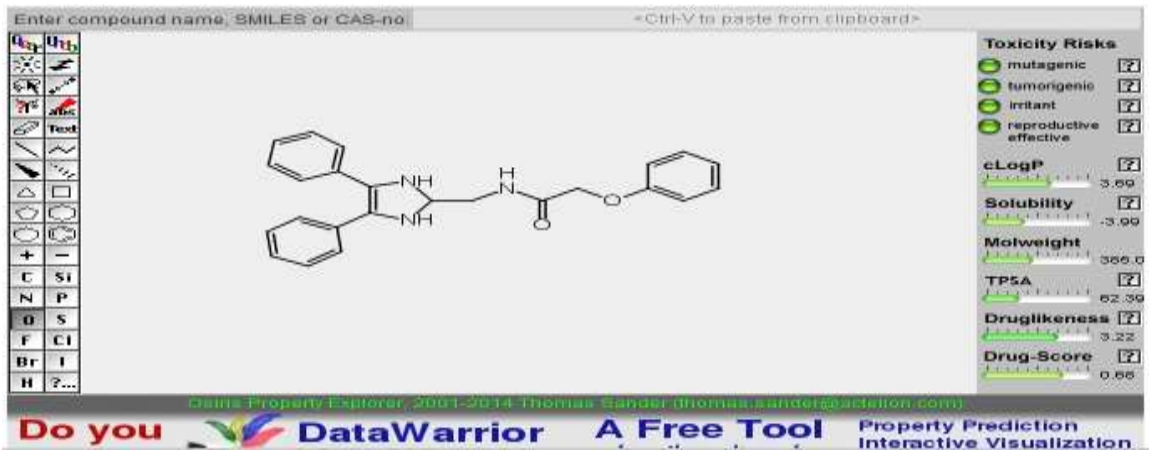


Fig.No.16: Biological activity, Physiochemical Properties and Toxicity profile images for Ligand 12

Thus the proposed ligands for synthesis Ligand (6), Ligand (9), Ligand (14), Ligand (13), Ligand (12) have satisfied all the above filtering method of good predictive activity with good docking scores and also drug likeness properties confirming that these molecules are accepted to be orally bioavailable.

V. B. SYNTHESIS AND CHARACTERIZATION

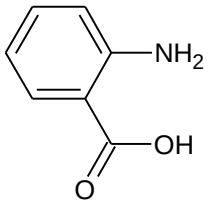
1. MATERIALS AND METHODS

SYNTHESIS

Reactants and solvents used were of analytical grade .Purification of the product were done by Thin layer chromatographic technique. TLC was performed using Aluminum plates pre-coated with Silica gel 60F 254 (E-Merck); detected using UV light chamber and Iodine chamber. Melting points were taken in an open capillary tubes and are present uncorrected.

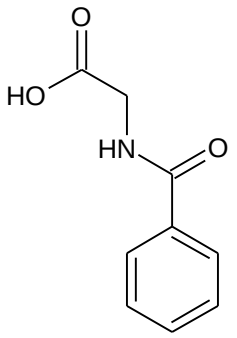
CHEMICAL USED FOR SYNTHESIS

ANTHRANILIC ACID



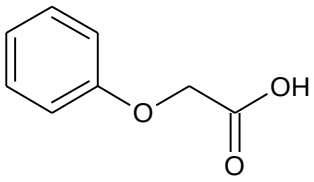
Molecular formula	**:**	$C_7H_7NO_2$
Molecular weight	**:**	137.14 g/mol.
Description	**:**	white solid
Melting point	**:**	144-148 °C
Boiling point	**:**	200 °C

HIPPURIC ACID



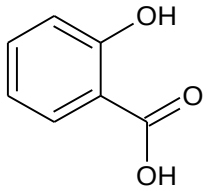
Molecular formula	**:**	$C_9H_9NO_3$
Molecular weight	**:**	179.17 g/mol.
Description	**:**	White crystals
Melting point	**:**	187-191 °C
Boiling point	**:**	240 °C

PHENOXY ACETIC ACID



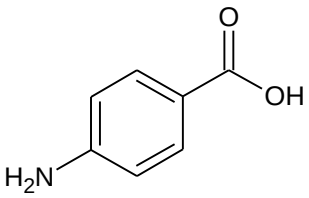
Molecular formula	**:**	$C_8H_8O_3$
Molecular weight	**:**	152.15 g/mol.
Description	**:**	White needle crystal
Melting point	**:**	98-100 °C
Boiling point	**:**	285 °C

SALICYLIC ACID



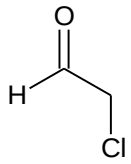
Molecular formula	**:**	$C_7H_6O_3$
Molecular weight	**:**	138.12 g/mol.
Description	**:**	white to off-white crystalline powder
Melting point	**:**	159 °C
Boiling point	**:**	211 °C

PARA AMINO BENZOIC ACID



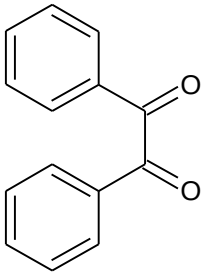
Molecular formula	**:**	$C_7H_7O_2N$
Molecular weight	**:**	137.14 g/mol.
Description	**:**	White Crystalline powder
Melting point	**:**	187 °C
Boiling point	**:**	340 °C

CHLORO ACETALDEHYDE



Molecular formula	**:**	C_2H_3ClO
Molecular weight	**:**	78.5 g/mol.
Description	**:**	Clear colorless liquid with pungent odour
Melting point	**:**	-28 to 23°C
Boiling point	**:**	80-100 °C

BENZIL



Molecular formula	**:**	$C_{14}H_{10}O_2$
Molecular weight	**:**	210.23 g/mol.
Description	**:**	Yellow solid
Melting point	**:**	94-95 °C
Boiling point	**:**	346 °C

INSTRUMENTS USED FOR CHARACTERIZATION

The synthesized compounds were characterized by Infra-red spectra (IR), Nuclear Magnetic Resonance (^{1}H NMR, ^{13}C NMR), GC-MASS Spectra.

Melting point

The melting point were recorded on a Melting point apparatus by using a capillary tube.

IR spectra

- IR Spectra was recorded using KBr pellets in then range 4000-500cm^{-1} on a ABB Bomen FT-IR spectrophotometer using KBr pellets

^{1}H NMR spectra

- Proton NMR (500MHz) Spectra was recorded were recorded deutrated methanol as solvent on BRUKER Advance III 500 NMR Spectrophotometer. Chemical shifts are shifted in parts per million downfield with reference to tetramethyl silane (TMS) as internal standard.

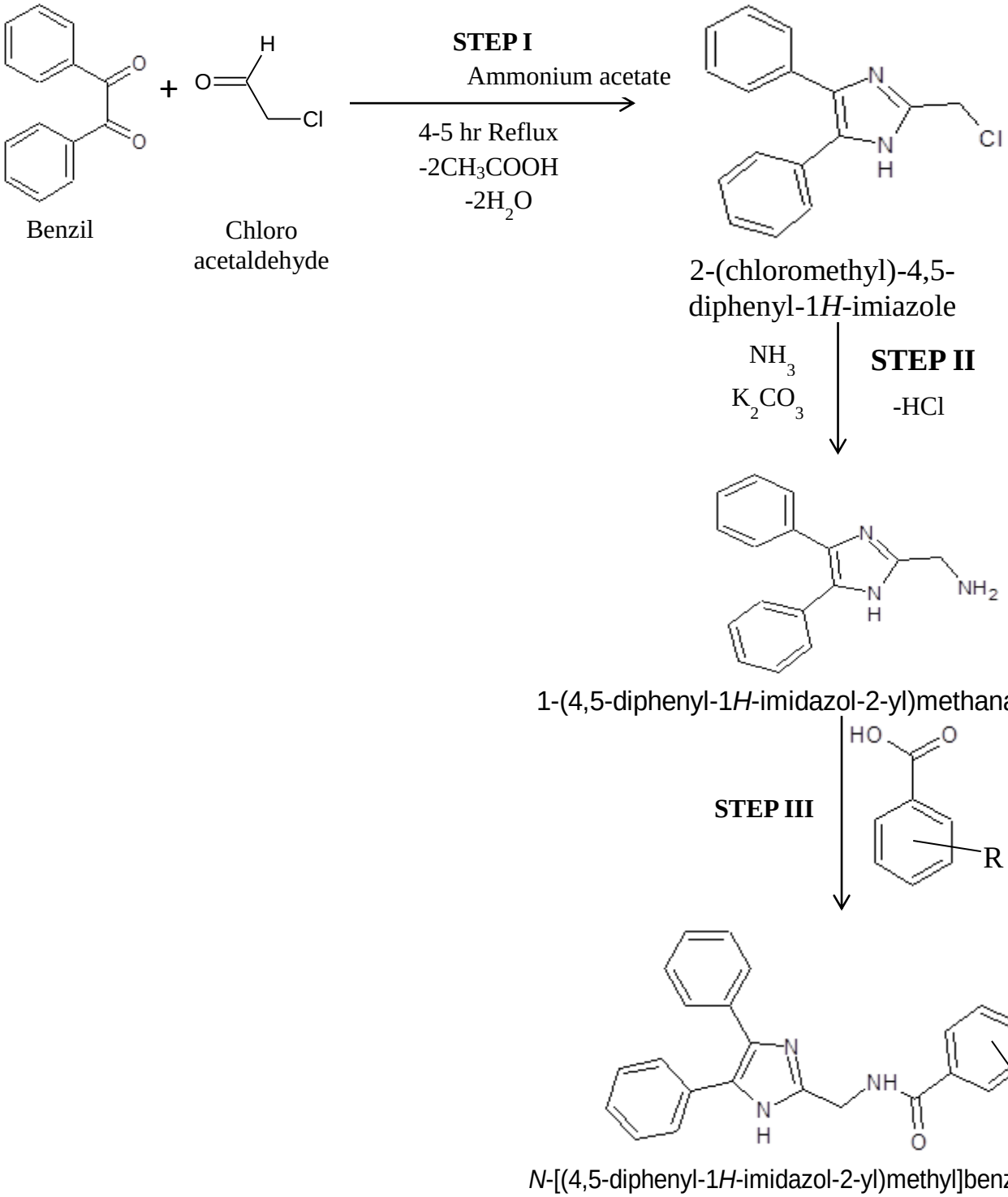
^{13}C NMR spectra

- ^{13}C-NMR was recorded using solvent $CDCl_3$ on BRUKER Advance III 400 NMR Spectrophotometer.

GC-MS spectra

GC-MS spectra were recorded using combined gas chromatography and mass spectroscopy on Perkin Elmer Clarus 680 GC-MS spectrometer.

CHEMICAL SYNTHESIS



R = 2-NH_2, 4-NH_2, 2-OH

Fig.No.17: Scheme

Procedure

STEP I: Synthesis of 2-substituted 4, 5-diphenyl imidazole 2-(chloromethyl)-4, 5-diphenyl-1*H*-imidazol (Radiswiski synthesis from benzil) [62].

Benzil (25 mmol, 5.25g), aldehyde (25 mmol) and ammonium acetate (10g) were dissolved in glacial acetic acid and then refluxed for 3-5 hrs. After refluxing, the reaction mixture was left overnight and filtered to remove any impurities that may be present. Water (300 ml) was then added to the filtrate and the precipitate formed was collected. The filtrate was neutralized with ammonium hydroxide and then the second crop of the solid was collected. The two crops of the solid were combined, dried and recrystallized from ethanol. The purity of product was established by single spot on TLC. The percentage yield was found to be 80 % w/w.

STEP II: 2-(chloromethyl)-4, 5-diphenyl-1*H*-imidazole from Step I product[63].

2-(chloromethyl)-4, 5-diphenyl-1*H*-imidazol (0.2 mol), was prepared by reported method. Ammonia (0.1 mol) in acetone-water mixture was added. K_2CO_3 (0.1mol, 14.0g) was added as an acid acceptor. The resulting mixture was refluxed for 3 hrs with occasional shaking. The resulting suspension was poured into ice cold water (500 ml) and then filtered. The solid product was collected and dried to give 2-(chloromethyl)-4, 5-diphenyl-1*H*-imidazole. The purity of product was established by single spot on TLC. The percentage yield was found to be 80 % w/w.

STEP III: *N*-[(4, 5-diphenyl-1*H*-imidazol-2-yl)methyl]-2-benzamide from step II process [64].

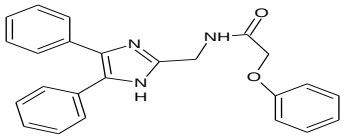
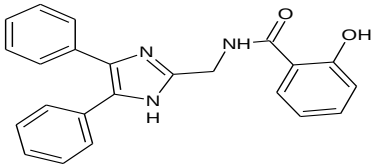
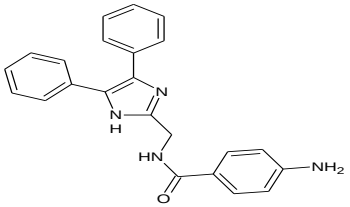
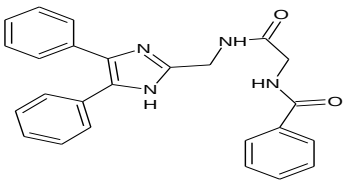
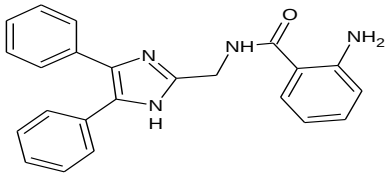
Aromatic acid (0.029 mol) and 2-(chloromethyl)-4, 5-diphenyl-1*H*-imidazol (0.026 mol), were dissolved in dry pyridine (0.25 mol) and refluxed for 8 hrs. Then the solution was cooled and poured in water. The separated mass was filtered, washed with water and dried. The products were recrystallized with ethanol. The purity of product was established by single spot on TLC. The percentage yield was found to be 80 % w/w.

2. RESULT AND DISSCUSSION

The imidazolyl derivatives were prepared in the same manner with different substituted Aromatic acids such as Para amino benzoic acid, Anthranilic acid, Hippuric acid, Phenoxy acetic acid, Salicylic acid.

List of the synthesized compounds structure and their IUPAC name is tabulated below,

Table.No.6: List of compounds synthesized

Drug code	IUPAC name	Structure of synthesized compounds
IPAA	*N*-[(4,5-diphenyl-1*H*-imidazol-2-yl)methyl]-2-phenoxyacetamide	
ISA	*N*-[(4,5-diphenyl-1*H*-imidazol-2-yl)methyl]-2-hydroxybenzamide	
IPABA	4-amino-*N*-[(4,5-diphenyl-1*H*-imidazol-2-yl)methyl]benzamide	
IHA	*N*-[(4,5-diphenyl-1*H*-imidazol-2-yl)methyl]-2-benzamide	
IAA	2-amino-*N*-[(4,5-diphenyl-1*H*-imidazol-2-yl)methyl]benzamide	

Physiochemical Properties of Synthesized Compounds

IPAA

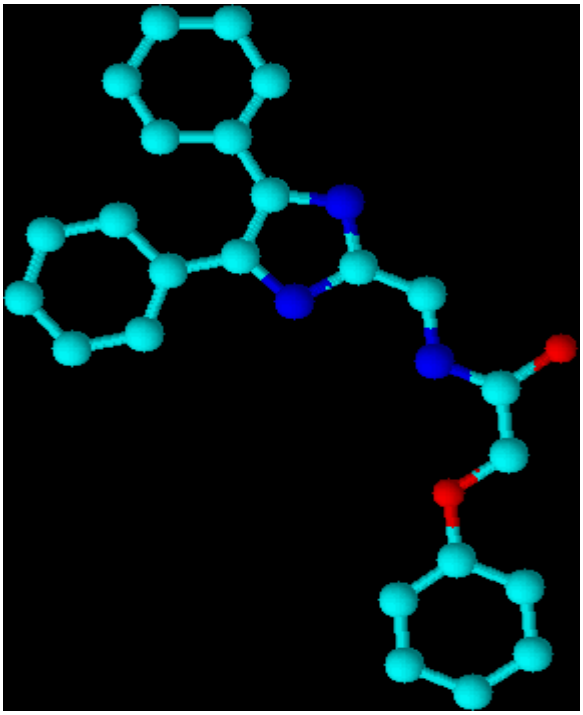


Fig.No.18: N-[(4, 5-diphenyl-1H-imidazol-2-yl) methyl]-2-phenoxyacetamide

Molecular Formula	:	C_{24} H_{21} N_3 O_2
Molecular Weight	:	384.44244
Composition	:	C (75.18 %) H (5.52%) N (10.96 %) O (8.35%)
Molar Refractivity	:	112.01 $\pm$ 0.3 cm^3
Molar Volume	:	315.2 $\pm$ 3.0 cm^3
Refractive Index (n)	:	1.629 $\pm$ 0.02
Density	:	1.216 $\pm$ 0.06 g /cm^3
Surface Tension (γ)	:	52.6 $\pm$ 3.0 dyne/cm
Dielectric Constant	:	N/A
Polarisability (α)	:	44.40 $\pm$ 0.5 cm^3
M+	:	383. 162828 Da
M-	:	383.163926 Da
[M+H]+	:	384.170635 Da
[M+H]-	:	384.171751 Da
[M-H]+	:	382.155003 Da
[M-H]-	:	382.1561 Da
Melting Point	:	45-70^oC
Rf Value	:	0.83
Yield	:	58.43 % w/w

ISA

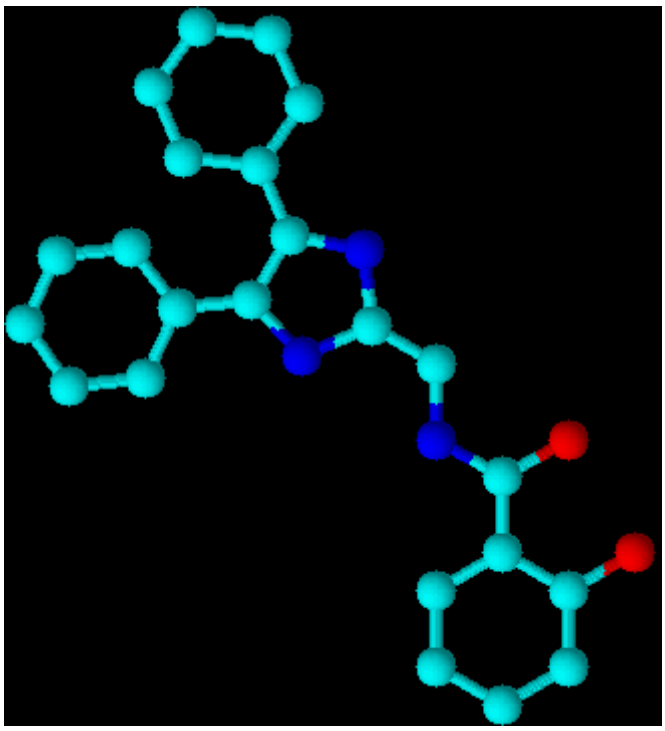


Fig.No.19: N-[(4, 5-diphenyl-1H-imidazol-2-yl) methyl]-2-hydroxybenzamide

Molecular Formula	:	C_{23} H_{19} N_3 O_2
Molecular Weight	:	369.41586
Composition	:	C (74.78 %) H (5.18%) N (11.37%) O (8.66%)
Molar Refractivity	:	107.95 $\pm$ 0.3 cm^3
Molar Volume	:	291.0 $\pm$ 3.0 cm^3
Refractive Index (n)	:	1.664 $\pm$ 0.02
Density	:	1.249 $\pm$ 0.06 g /cm^3
Surface Tension (γ)	:	59.4 $\pm$ 3.0 dyne/cm
Dielectric Constant	:	N/A
Polarisability (α)	:	42.79 $\pm$ 0.5 cm^3
M+	:	369. 147178 Da
M-	:	369.148275 Da
[M+H]+	:	370.155003 Da
[M+H]-	:	370.1561 Da
[M-H]+	:	368.139353 Da
[M-H]-	:	368.14045 Da
Melting Point	:	55-75^oC
Rf Value	:	0.83
Yield	:	59.24 % w/w

IPABA

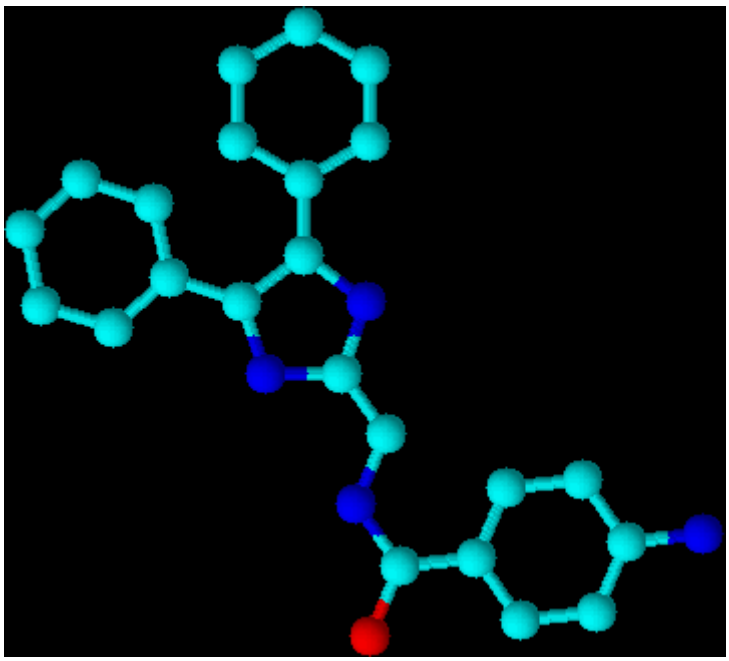


Fig.No.20: 4-amino-N-[(4, 5-diphenyl-1H-imidazol-2-yl) methyl] benzamide

Molecular Formula	:	C_{23} H_{20} N_4 O
Molecular Weight	:	368.4311
Composition	:	C (74.98 %) H (5.47%) N (15.21%) O (4.34%)
Molar Refractivity	:	110.30 $\pm$ 0.3 cm^3
Molar Volume	:	294.8 $\pm$ 3.0 cm^3
Refractive Index	:	1.671 $\pm$ 0.02
Density	:	1.249 $\pm$ 0.06 g /cm^3
Surface Tension (γ)	:	59.4 $\pm$ 3.0 dyne/cm
Dielectric Constant	:	N/A
Polarisability (α)	:	43.72 $\pm$ 0.5 cm^3
M+	:	368. 163163 Da
M-	:	368.16426 Da
[M+H]+	:	369. 170988 Da
[M+H]-	:	369.172085 Da
[M-H]+	:	369.155338 Da
[M-H]-	:	367. 156435 Da
Melting Point	:	50-80^{o}C
Rf Value	:	0.54
Yield	:	78.3284 % w/w

IHA

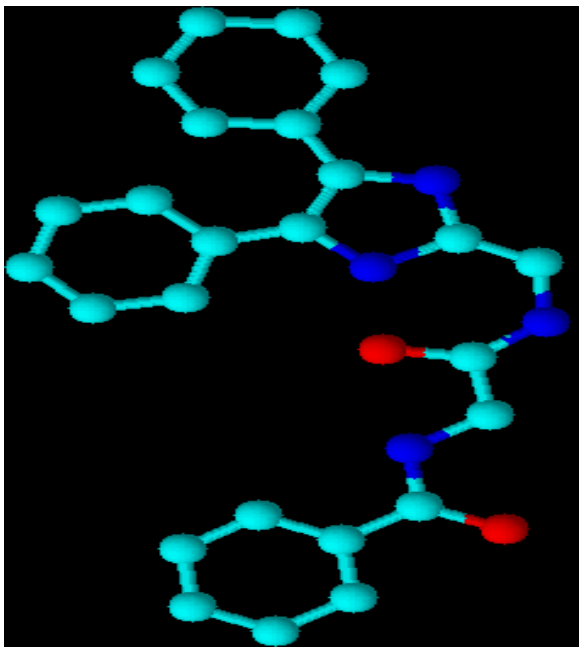


Fig.No.21: N-[(4, 5-diphenyl-1H-imidazol-2-yl) methyl]-2-benzamide

Molecular Formula	:	C_{25} H_{22} N_4 O_2
Molecular Weight	:	410.46778
Composition	:	C (73.15 %) H (5.40%) N (13.65 %) O (7.80%)
Molar Refractivity	:	118.96 $\pm$ 0.3 cm^3
Molar Volume	:	331.5 $\pm$ 3.0 cm^3
Refractive Index (n)	:	1.636 $\pm$ 0.02
Density	:	1.238 $\pm$ 0.06 g /cm^3
Surface Tension (γ)	:	55.6 $\pm$ 3.0 dyne/cm
Dielectric Constant	:	N/A
Polarisability (α)	:	47.16 $\pm$ 0.5 cm^3
M+	:	410. 173727 Da
M-	:	410.174825 Da
[M+H]+	:	411.181552 Da
[M+H]-	:	411.18265 Da
[M-H]+	:	409.165902 Da
[M-H]-	:	409.167 Da
Melting Point	:	70-85^oC
Rf Value	:	0.80
Yield	:	57.31 % w/w

IAA

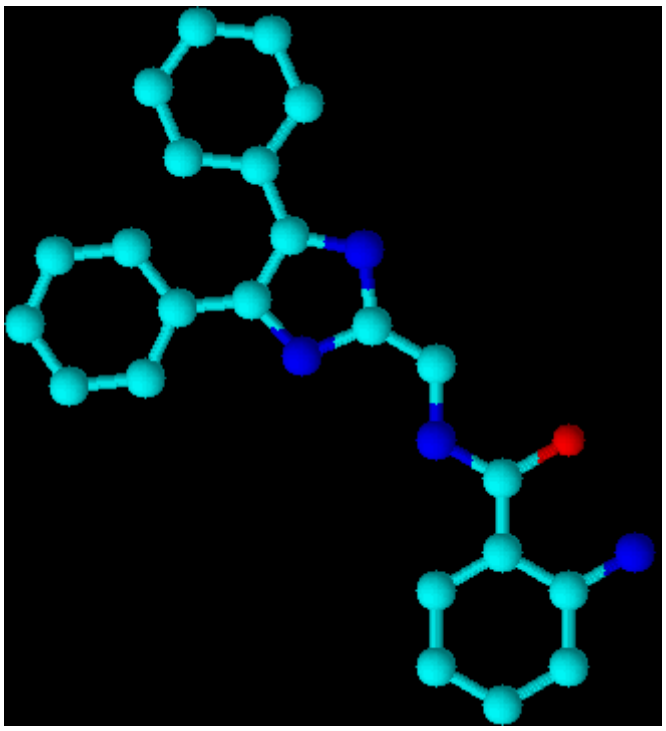


Fig.No.22: 2-amino-N-[(4, 5-diphenyl-1H-imidazol-2-yl)methyl] benzamide

Molecular Formula	:	C_{23} H_{20} N_4 O
Molecular Weight	:	368.4311
Composition	:	C (74.98 %) H (5.47%) N (15.21%) O (4.34%)
Molar Refractivity	:	110.30 $\pm$ 0.3 cm^3
Molar Volume	:	294.8 $\pm$ 3.0 cm^3
Refractive Index	:	1.671 $\pm$ 0.02
Density	:	1.249 $\pm$ 0.06 g /cm^3
Surface Tension (γ)	:	59.4 $\pm$ 3.0 dyne/cm
Dielectric Constant	:	N/A
Polarisability (α)	:	43.72 $\pm$ 0.5 cm^3
M+	:	368. 163163 Da
M-	:	368.16426 Da
[M+H]+	:	369. 170988 Da
[M+H]-	:	369.172085 Da
[M-H]+	:	369.155338 Da
[M-H]-	:	367. 156435 Da
Melting Point	:	60-85^oC
Rf Value	:	0.77
Yield	:	57.36 % w/w

3. CHARACTERIZATION

All the synthesized compounds IPAA, ISA, IPABA, IHA, IAA have been characterized by using different analytical techniques like IR, NMR, GC-MS and the results are given below,

IR spectra of compound IPAA

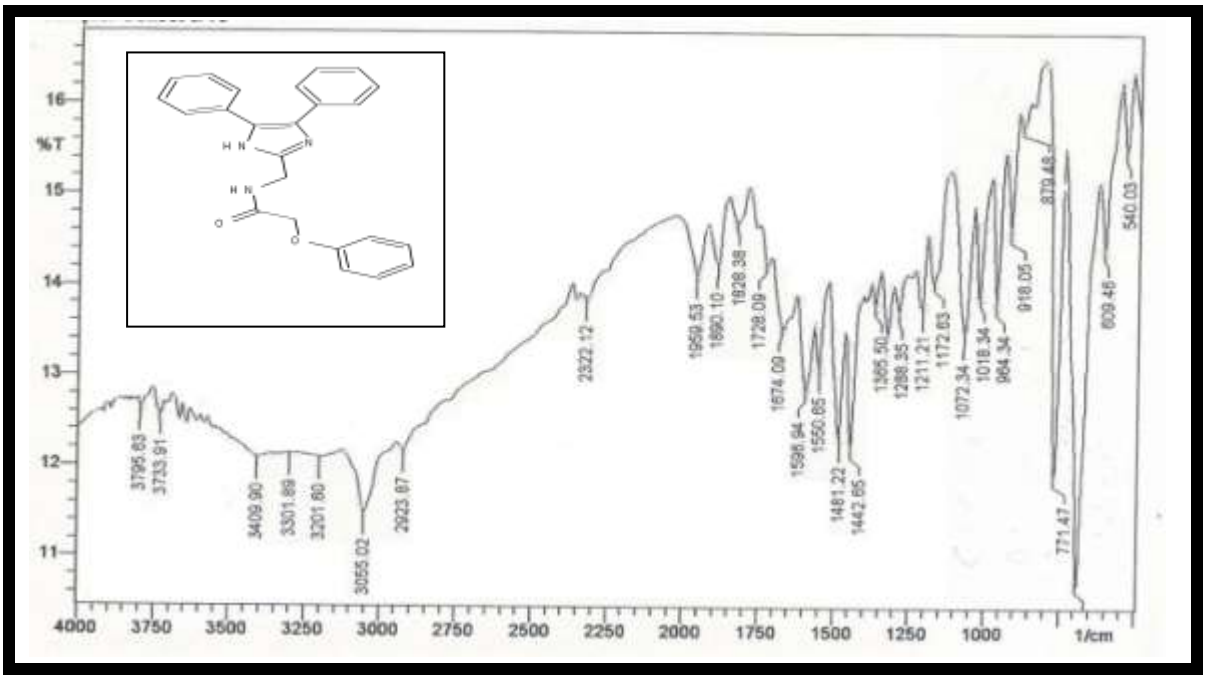


Table.No.7: IR Spectral data of synthesized IPAA (KBr)

S. No	Wave Number (Cm^{-1})	Functional Group
1.	3733.91	Amide NH str
2.	3409.90	Hetero -Ar NH str
3.	3055.02	Ar CH str
4.	2923.44	Alkyl CHstr
5.	1674.09	Amide C=O str
6.	1596.94	Imidazole C=N str
7.	1481.22	C-N str
8.	1442.65	Phenoxy C-O str
9.	771.47	Ar CH bending
10.	694.22	Alkyl CH bending

IR spectra of compound ISA

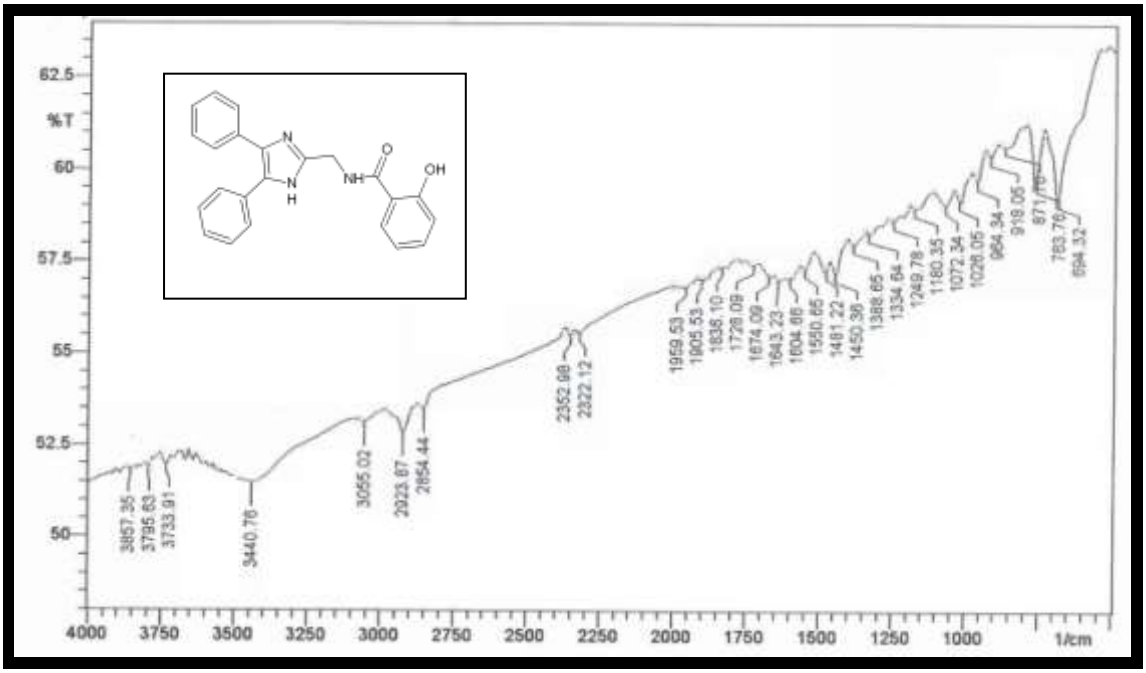


Table.No.8: IR Spectral data of ISA (KBr)

S. No	Wave Number (Cm^{-1})	Functional Group
1	3795.63	Amide NH str
2	3733.91	Hetero Ar NH str
3	3440.76	Phenolic OH str
4	2923.87	Ar CH str
5	2854.44	Alkyl CH str
6	1674.09	Amide C=O str
7	1643.23	Imidazole C=N str
8	1481.22	C-N str
9	1450.36	Phenolic O-H bending
10	1072.34	C-C bending
11	763.76	Ar CH bending
12	694.32	Alkyl CH bending

IR spectra of compound IPABA

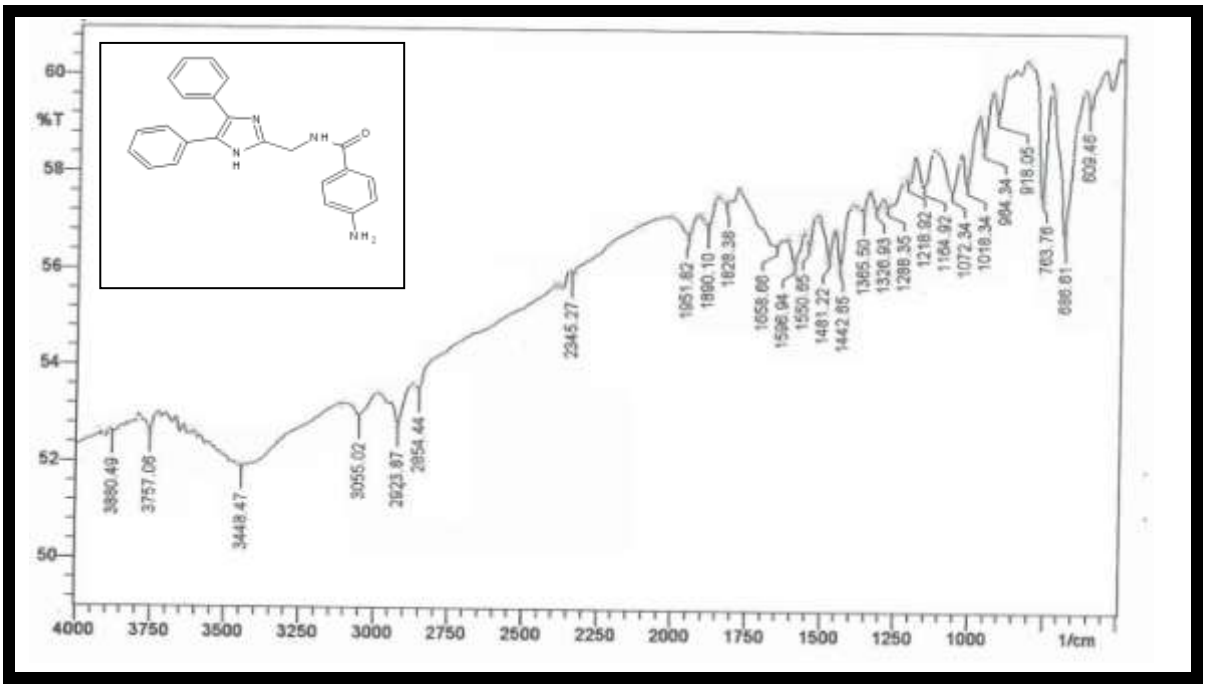


Table.No.9: IR Spectral data of IPABA (KBr)

S. No	Wave Number (Cm^{-1})	Functional Group
1	3757.06	Amide NH str
2	3448.47	Hetero Ar NH str
3	2923.87	Ar CH str
4	2854.44	Alkyl CH str
5	1658.66	Amide C=O str
6	1596.94	Imidazole C=N str
7	1481.22	C-N str
8	1365.50	Ar NH bending
9	1072.34	C-C bending
10	763.73	Ar CH bending
11	686.61	Alkyl CH bending

IR spectra of compound IHA

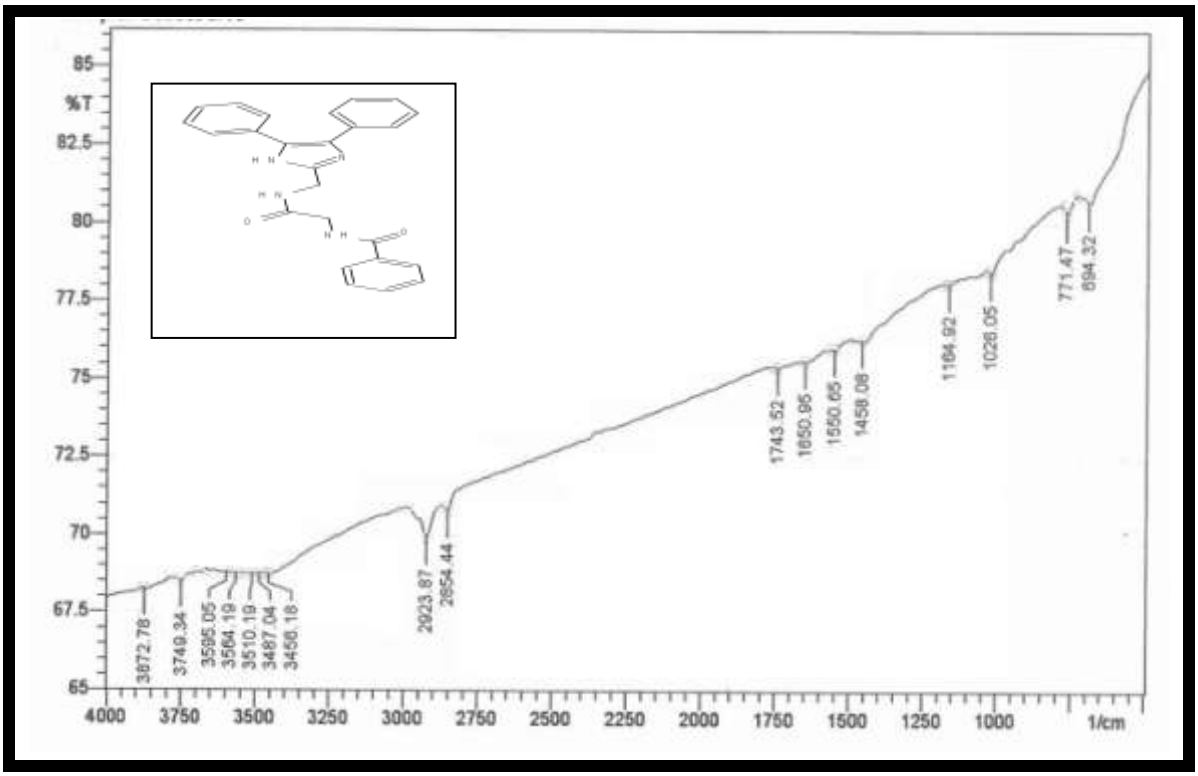


Table.No.10: IR Spectral data of IHA (KBr)

S. No	Wave Number (Cm^{-1})	Functional Group
1	3749.34	Amide NH str
2	3487.04	Hetero Ar NH str
3	2923.87	Ar CH str
4	2854.44	Alkyl CH str
5	1650.95	Amide C=O str
6	1550.65	Imidazole C=N str
7	1458.08	C-N str
8	1164.92	C-C str
9	771.47	Ar CH bending
10	694.32	Alkyl CH bending

IR spectra of compound IAA

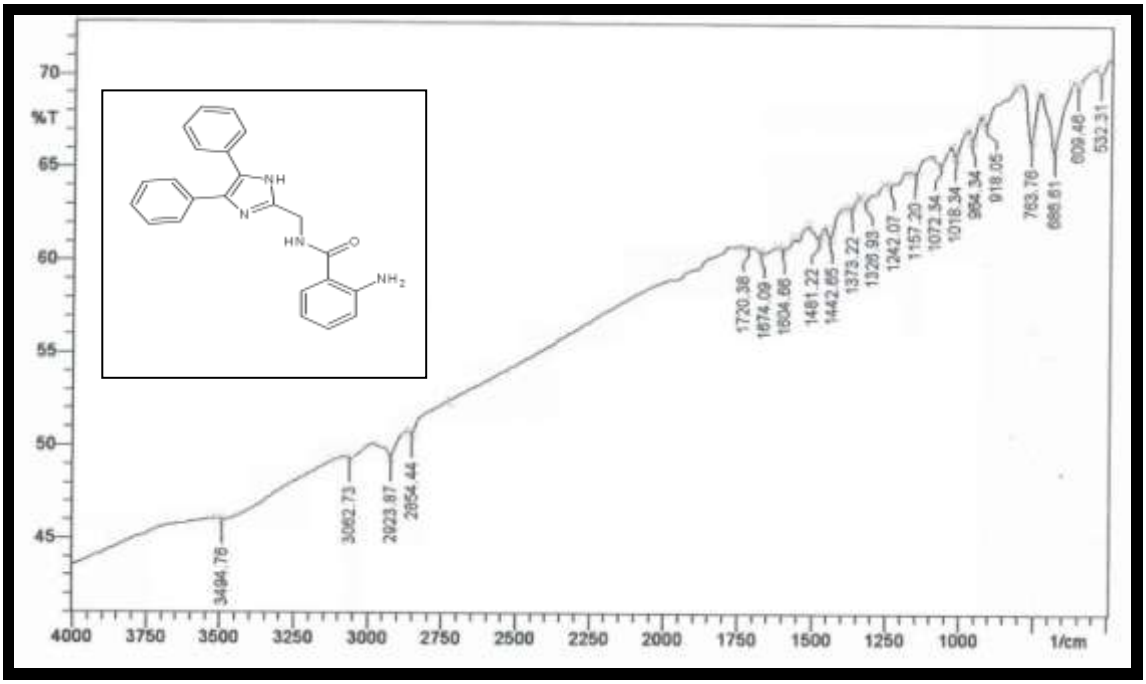


Table.No.11: IR Spectral data of IAA (KBr)

S. No	Wave Number (Cm^{-1})	Functional Group
1	3733	Amide NH str
2	3494.76	Hetero Ar NH str
3	2923.87	Ar CH str
4	2854.44	Alkyl CH str
5	1674.09	Amide C=O str
6	1604.66	Imidazole C=N str
7	1481.22	C-N str
8	1373.22	Ar NH bending
9	1072.34	C-C bending
10	763.76	Ar CH bending
11	636.81	Alkyl CH bending

^{1}H NMR spectra of compound IPAA

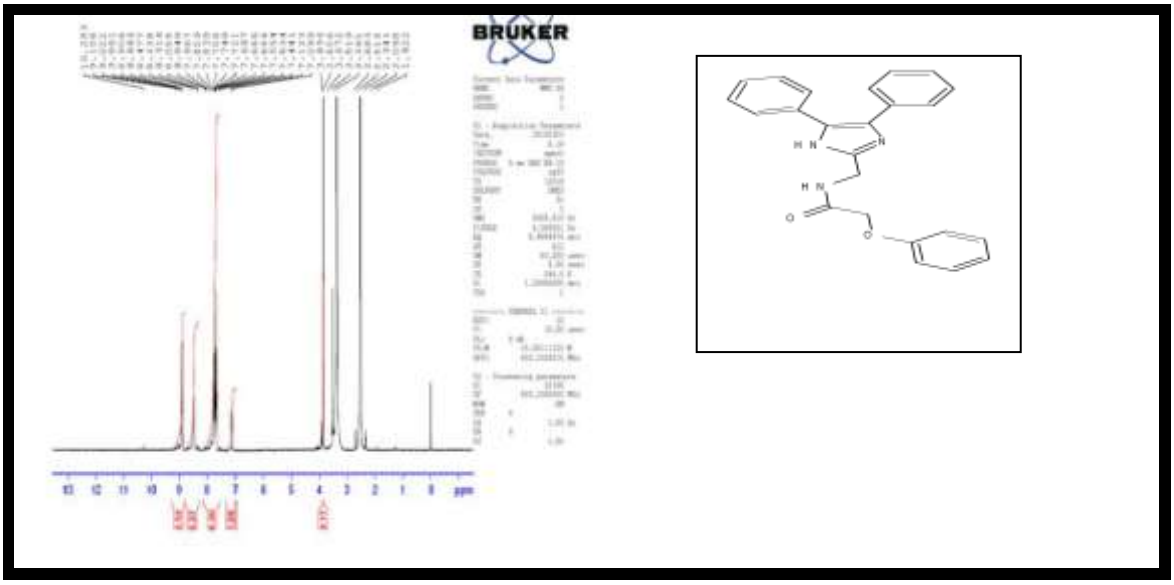


Table.No.12: ^{1}HNMR Spectral nature of IPAA

S. No	δ value	Nature of peak	Number of proton
1	4	Multiplet	4 proton
2	8	Doublet	2 proton
3	7-9	Multiplet	15 proton

^{1}H NMR spectra of compound ISA

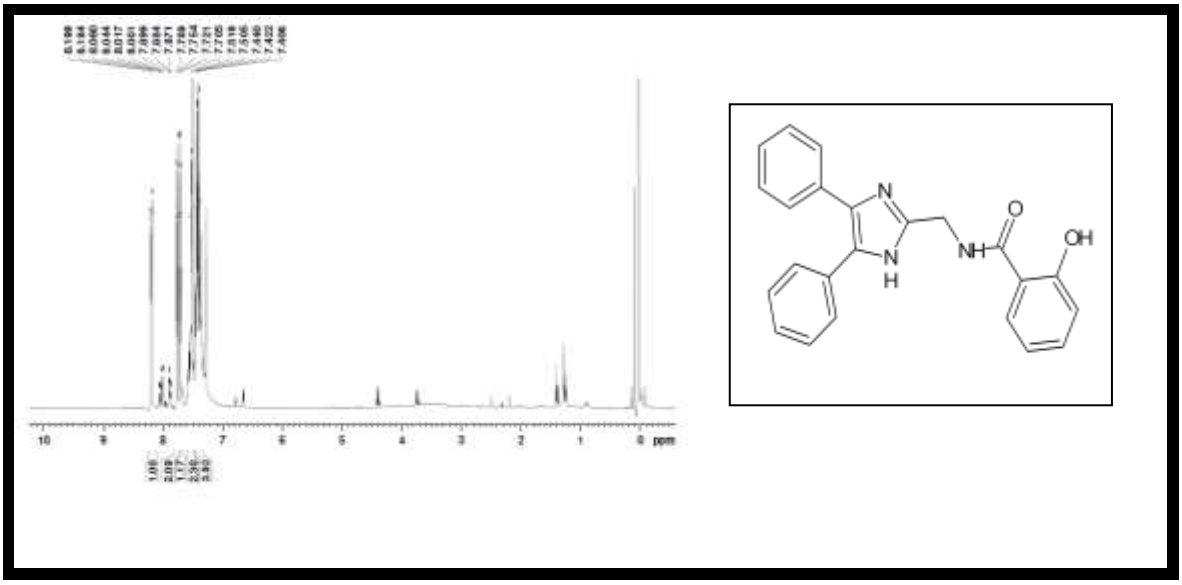


Table.No.13: ^{1}HNMR Spectral nature of ISA

S. No	δ value	Nature of peak	Number of proton
1	8	Multiplet	7 proton
2	7-9	Multiplet	15 proton

^{1}H NMR spectra of compound IPABA

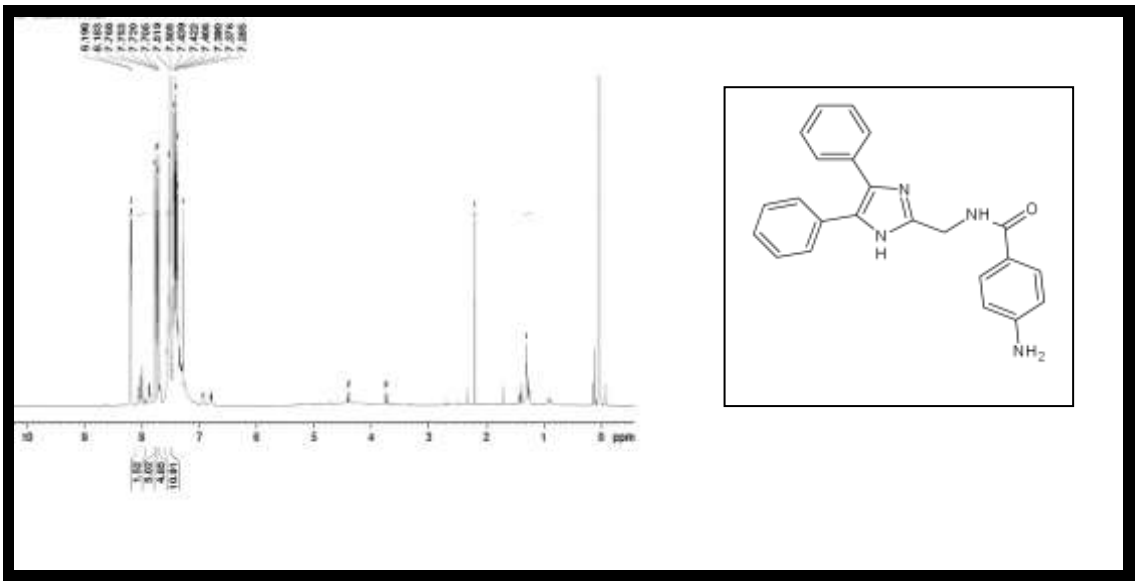


Table.No.14: ^{1}HNMR Spectral nature of IPABA

S. No	δ value	Nature of peak	Number of proton
1	8	Triplet	3 proton
2	7-9	Multiplet	17 proton

^{1}H NMR spectra of compound IHA

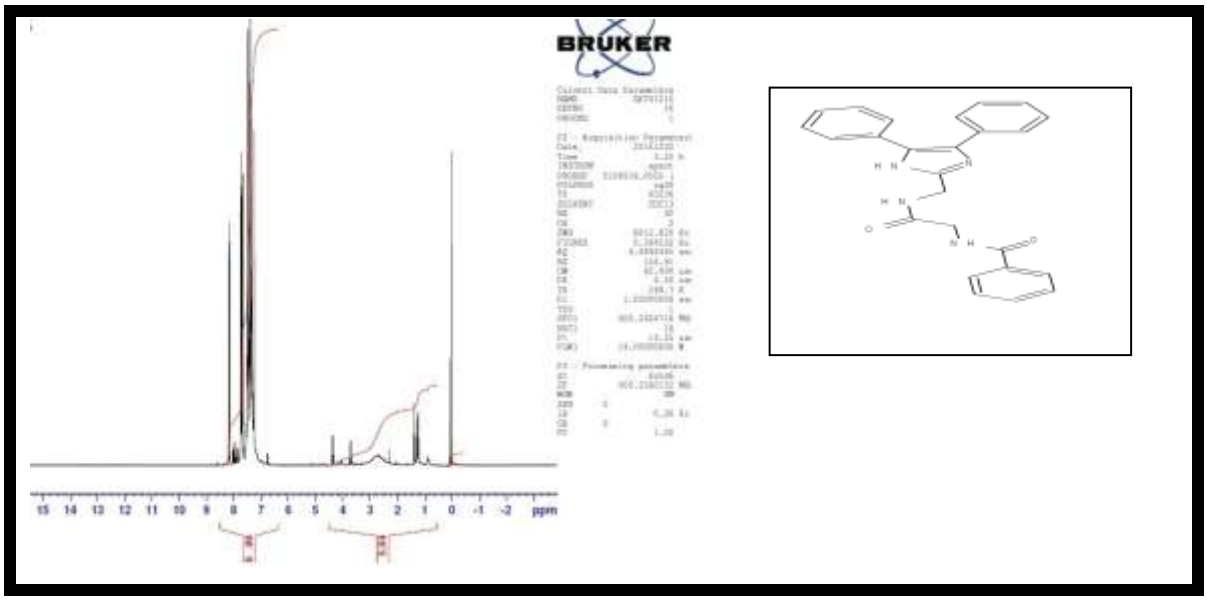


Table.No.15: ^{1}HNMR Spectral nature of IHA

S. No	δ value	Nature of peak	Number of proton
1	4.46	Triplet	3 proton
2	8	Multiplet	3 proton
3	7-9	Multiplet	16 proton

^{1}H NMR spectra of compound IAA

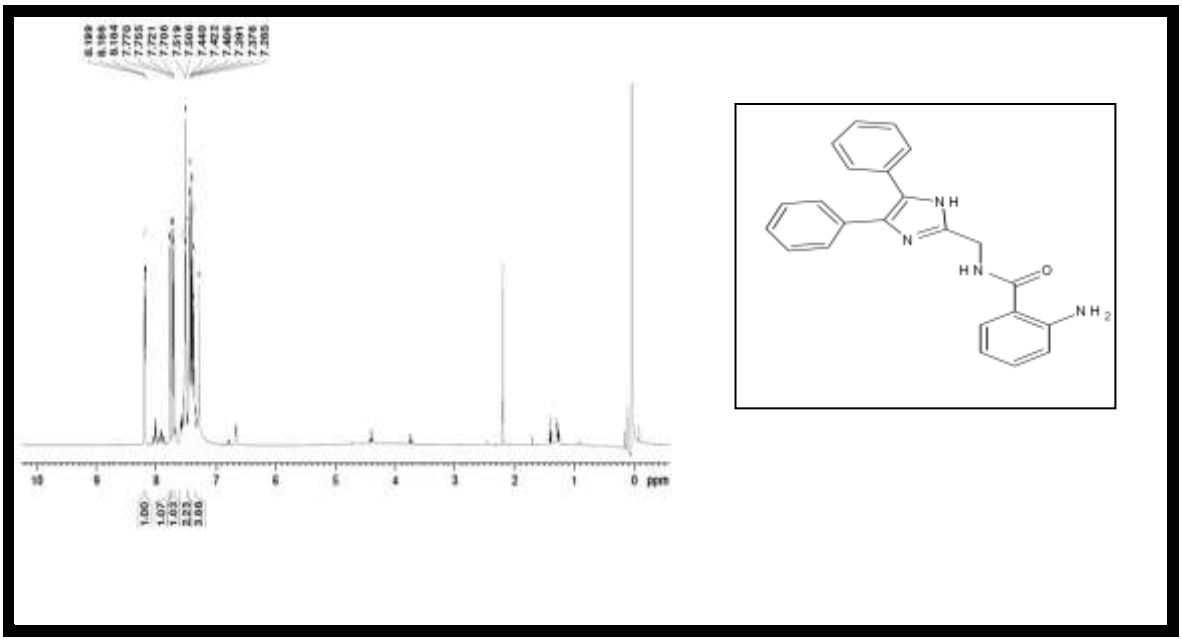


Table.No.16: ^{1}HNMR Spectral nature of IAA

S. No	δ value	Nature of peak	Number of proton
1	8	Triplet	3 proton
2	7-9	Multiplet	17 proton

^{13}C NMR spectra of compound IPAA

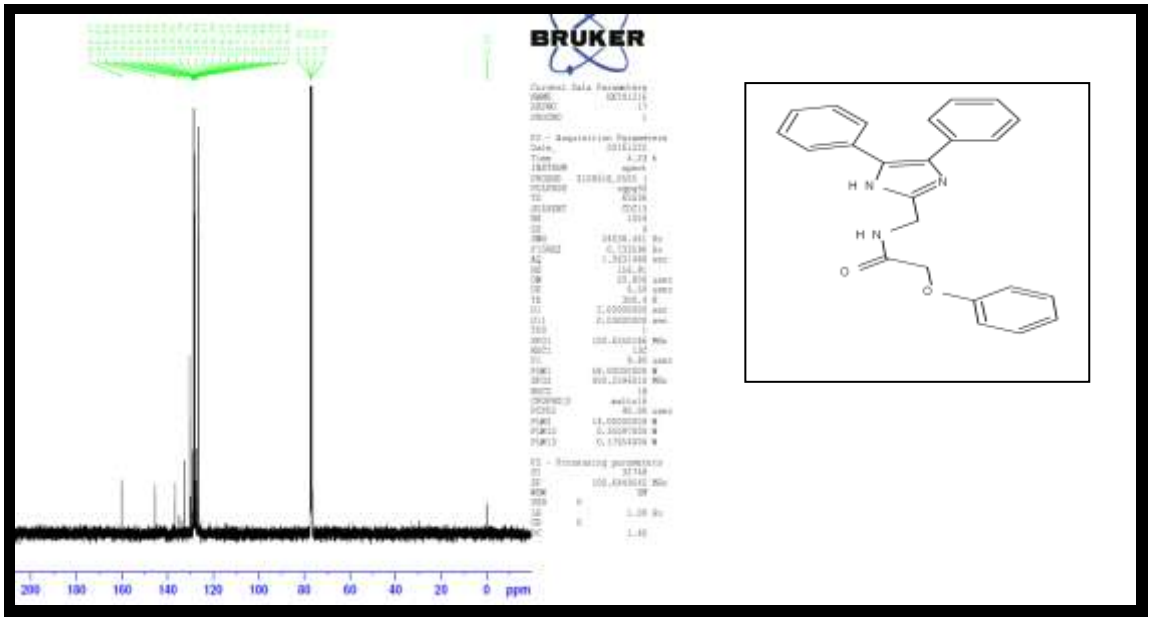


Table.No.17:* ^{13}CNMR *Spectral nature of IPAA

S. No	δ Value	Functional carbon
1	129.4	Imidazole C (3)
2	133.1	Aromatic C (16)
3	37.9	Alkyl C (2)
4	169.0	Amide C (1)

^{13}C NMR spectra of compound ISA

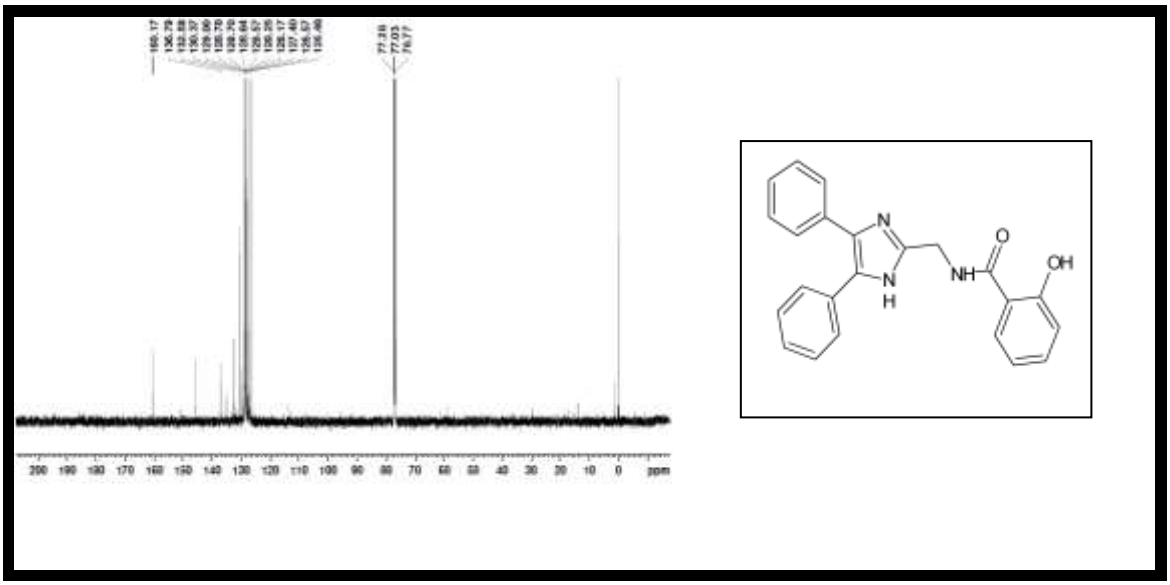


Table.No.18:* ^{13}CNMR *Spectral nature of ISA

S. No	δ Value	Functional carbon
1	129.4	Imidazole C (3)
2	133.1	Aromatic C (16)
3	37.9	Alkyl C (1)
4	169.0	Amide C (1)

^{13}C NMR spectra of compound IPABA

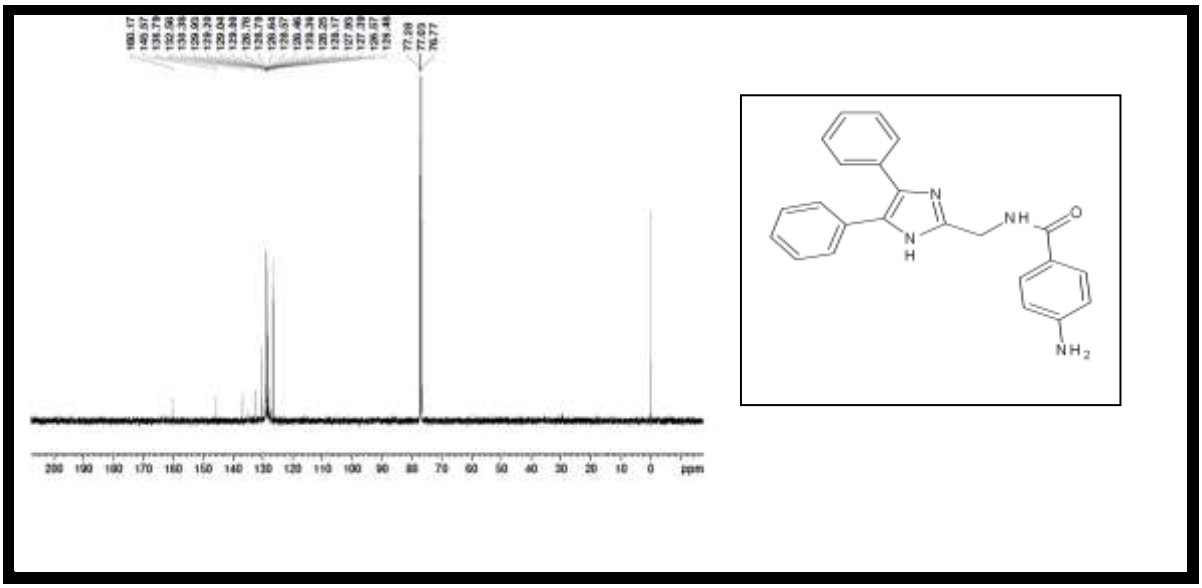


Table.No.19: **^{13}CNMR** *Spectral nature of IPABA*

S. No	δ Value	Functional carbon
1	129.4	Imidazole C (3)
2	133.1	Aromatic C (16)
3	37.9	Alkyl C (1)
4	169.0	Amide C (1)

^{13}C NMR spectra of compound IHA

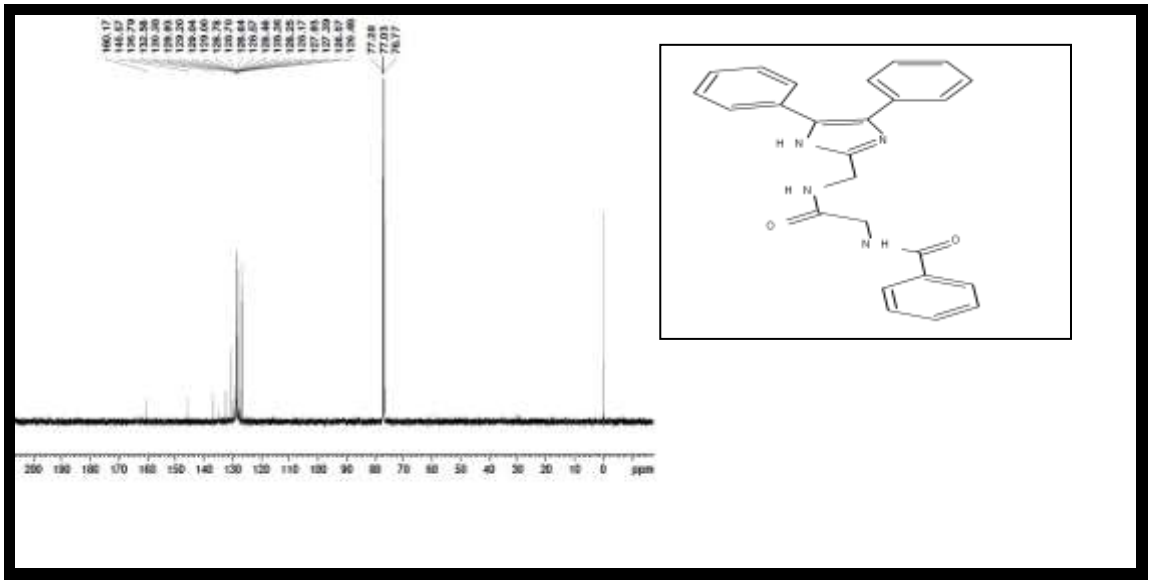


Table.No.20: **^{13}CNMR** *Spectral nature of IHA*

S. No	δ Value	Functional carbon
1	129.4	Imidazole C (3)
2	133.1	Aromatic C (16)
3	37.9	Alkyl C (2)
4	169.0	Amide C (2)

^{13}C NMR spectra of compound IAA

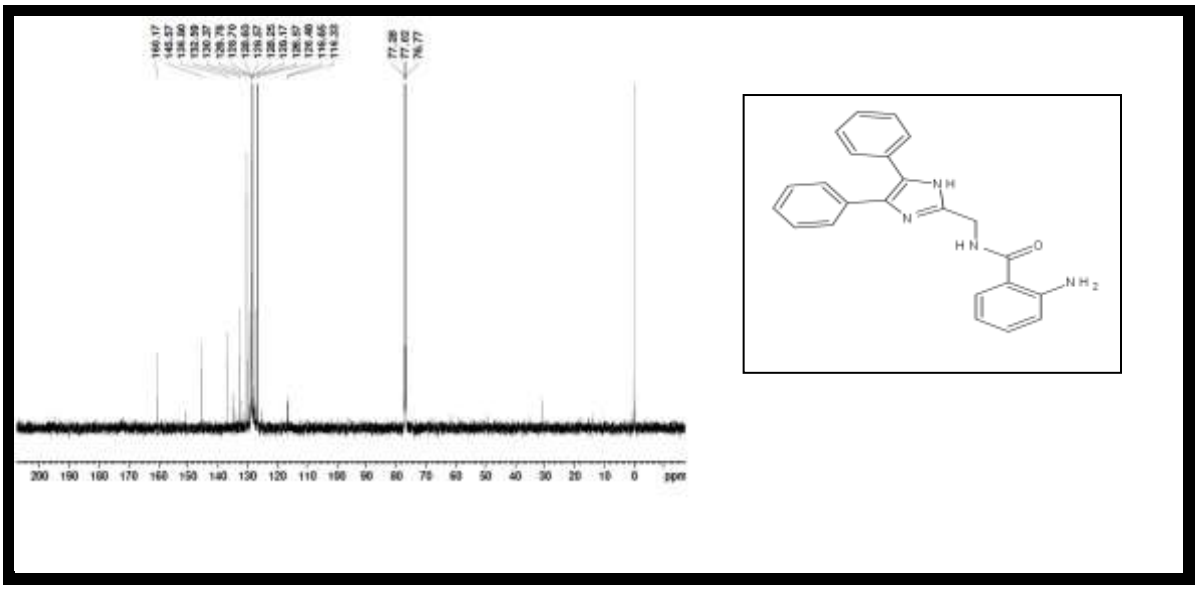
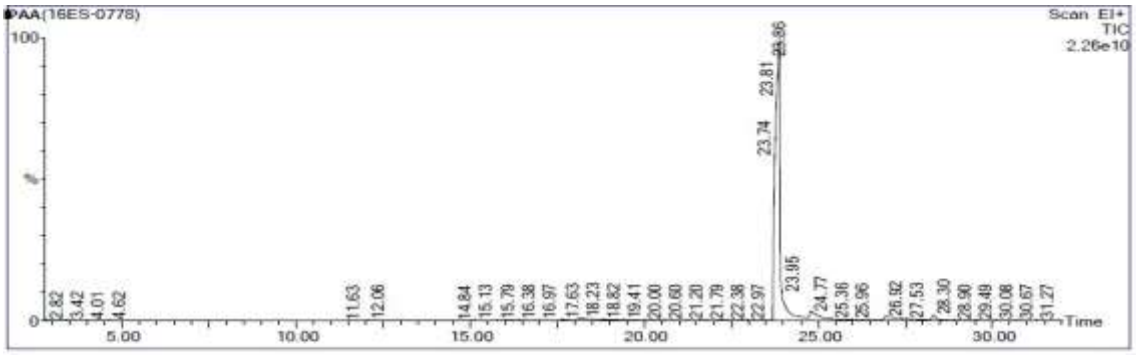


Table.No.21: **^{13}CNMR** *Spectral nature of IAA*

S. No	δ Value	Functional carbon
1	129.4	Imidazole C (3)
2	133.1	Aromatic C (16)
3	37.9	Alkyl C (1)
4	169.0	Amide C (1)

GC-MS Spectra of compound IPAA



#	RT	Scan	Height	Area	Area %	Norm %
1	23.892	4217	22,398,365,696	3,590,128,384.0	93.421	100.00

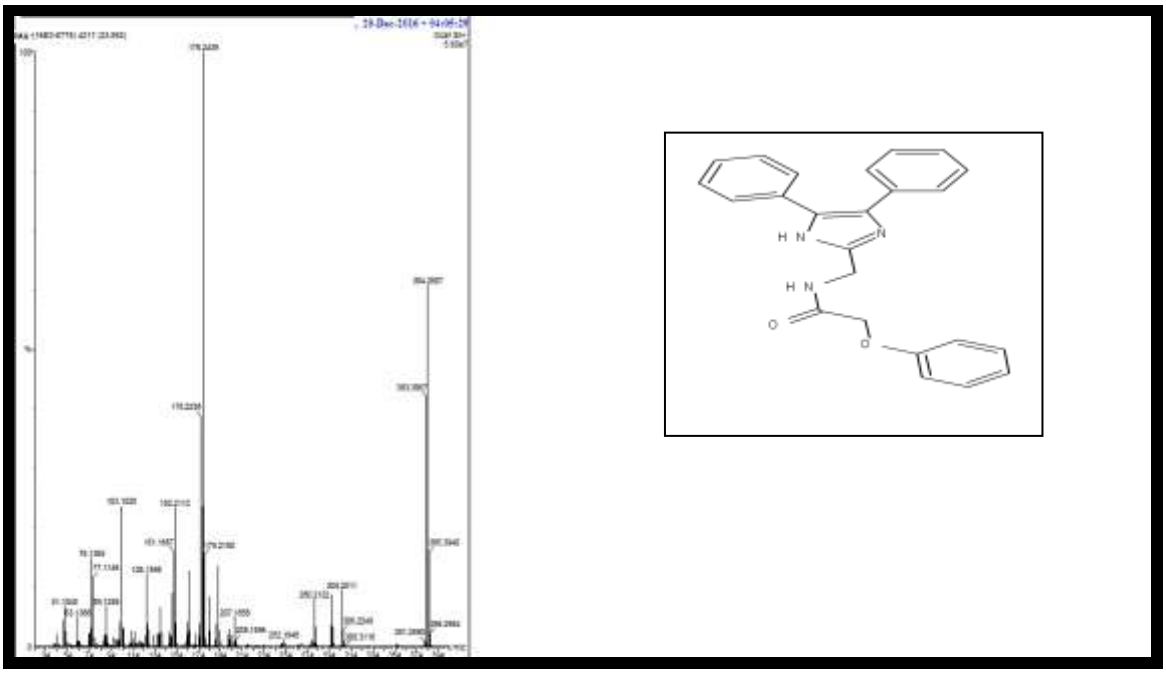


Table.No.22: **GC-MS** *Spectral nature of compound IPAA*

S. No	Peak	Molecular Weight
1	M	384.2807
2	M+1	385.3940
3	M-1	383.3067

4. DOCKING STUDIES OF SYNTHESIZED COMPOUNDS

a) Rigid docking using *GLIDE 10.2* version

Crystal structure of *BTK* (PDB ID: 5FBN) was used for the study. Structure-based docking studies were carried out using GLIDE version 10.2 .The protein 3D structure was downloaded from the protein databank (PDB), the solvent molecules in the protein were removed and hydrogen atoms were added to the protein using Cerius2 module . . Fifty distinct poses of each synthesized compounds IAA, IPAA, IHA, ISA, IPABA were generated and docked against the hydrophobic binding pocket of *Butyro tyrosine kinase* Tyr 461, Asp 426, Phe 540,Asp539 and Thr 474 of *BTK* active site. Glide fitness scores were found to correlate well with the biological activities it was also observed that hydrogen bond interactions play a major role in deciding the fitness score of the molecule.

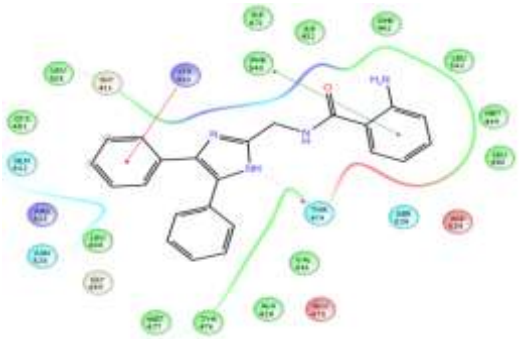
b) Flexible docking using *Argus lab 4.1*

FlexibleDocking was performed on 33 BTK inhibitors using *Argus 4.1* the binding region was defined using a grid 32 X 32 X 32 box centered on the centroid of the target. Default setting were used for all the remaining parameters. Fifty distinct poses of each synthesized compounds IAA, IPAA, IHA, ISA, IPABA were generated. The hydrophobic binding pocket of *Butyro tyrosine kinase* is made up of key residues Tyr 461, Asp 426 and Thr 474 was considered as active site for docking and saved the docked protein ligand complexes as brook haven pdb.files (*.pdb).Inspecting docking results consisting of high scoring poses found by Molegro molecular viewer. A better understanding of the interactions is obtained by viewing the molecules in the active site.

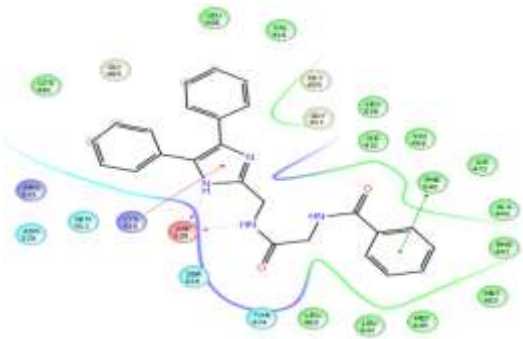
RESULTS AND DISCUSSIONS

All the synthesized compounds IAA, IPAA, IHA, ISA, IPABA were docked against enzyme target *BTK* (PDB ID: 5FBN) to study their binding interactions by using *GLIDE 10.2* and snapshots, docking results were given below

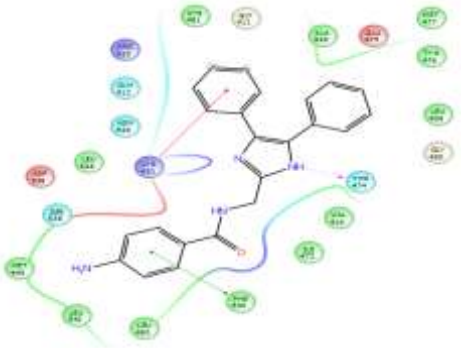
IAA



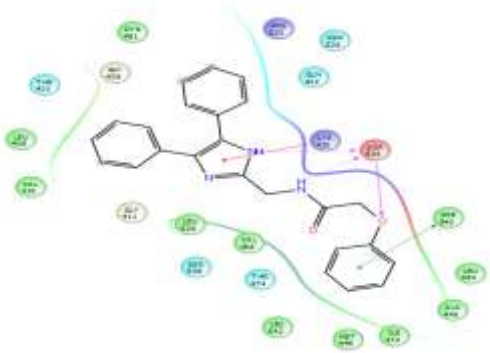
IHA



IPABA



IPAA



ISA

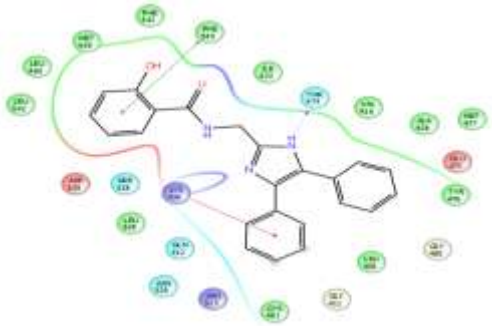
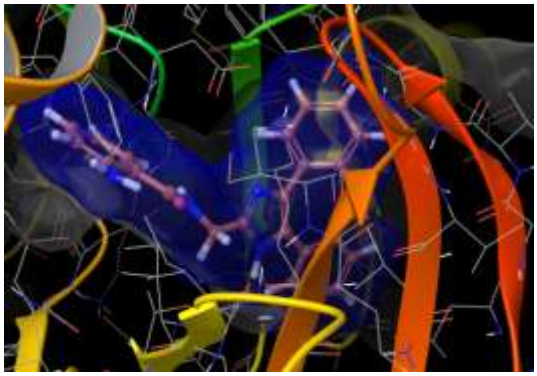
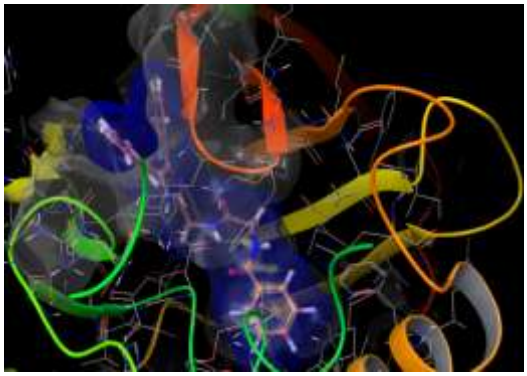


Fig.No.23: The binding conformation and Hydrogen bonding, pi-pi static interaction of docked ligands

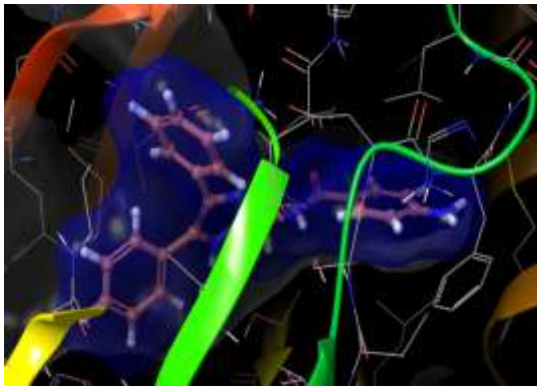
IAA



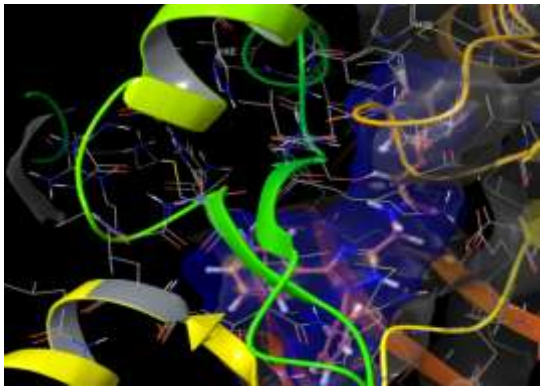
IHA



IPABA



IPAA



ISA

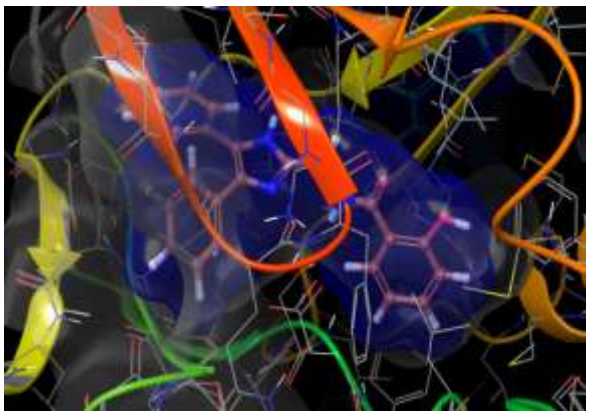


Fig.No.24: The binding mode of the designed ligands in the active sites of BTK. The BTK protein is shown in ribbon model while the ligands were depicted by sticks model.

Table.No.23: Docking Score of synthesized compounds using Glide 10.2

S.no	Compound code	Docking score (SP - Glide 10.2)	Energy of protein KJ/mol	Minimized energy of protein KJ/mol
1	IPABA	-6.808	-80.7430	-177.6756
2	ISA	-6.437	39.506	-39.6756
3	IAA	-7.632	25.8316	-41.4503
4	IHA	-7.770	-155.253	-155.2529
5	IPAA	-7.970	-33.3296	-115.6136

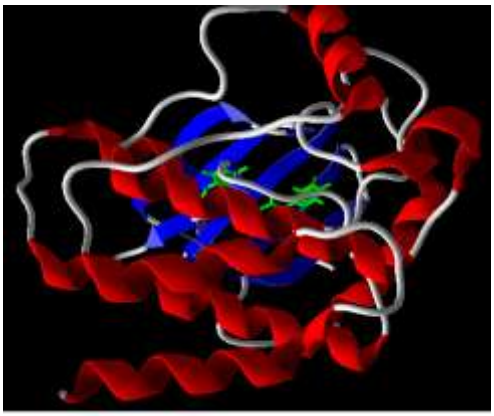
Table.No.24: Type of interaction between ligand and the target proteins

S.no	Compound code	Number of interaction	Functional group involved in interaction
1	IPABA	1. Hydrogen bond 2. Pi-Pi static	Imidazole NH with Thr 474 Substituent of NH with Phe 540
2	ISA	1. Hydrogen bond 2. Pi-Pi static	Imidazole NH with Thr 474 Aromatic acid with Phe 540
3	IAA	1. Hydrogen bond 2. Pi-Pi static	Imidazole NH with Thr 474 Substituent of NH with Phe 540
4	IHA	1. Hydrogen bond 2. Pi-Pi static	Imidazole NH with Asp 539 Phenyl substituted imidazole with Phe 540
5	IPAA	1. Hydrogen bond 2. Pi-Pi static	Imidazole NH, substituted NH, O from aromatic acid with Asp 539 Aromatic acid with Phe 540

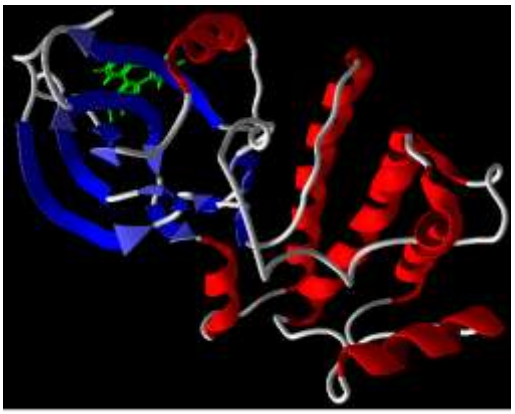
DOCKING STUDIES BY USING *ARGUS LAB 4.1*

All the synthesized compounds IAA, IPAA, IHA, ISA, IPABA were docked against enzyme target *BTK* (PDB ID: 5FBN) to study their binding interactions by using *Argus lab 4.1* and snapshots ,docking results were given below

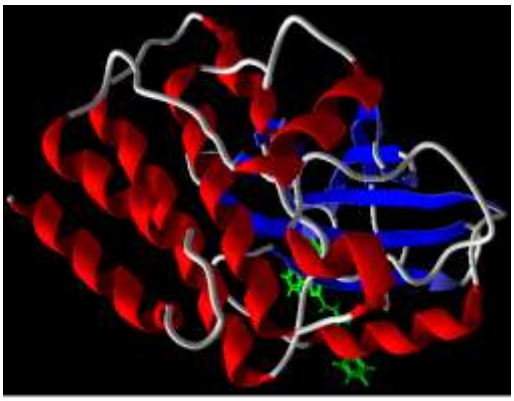
IAA



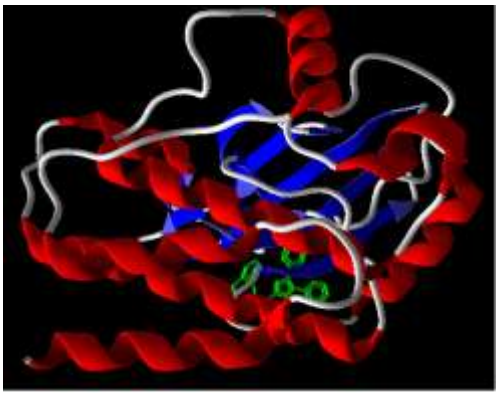
IPABA



IHA



IPAA



ISA

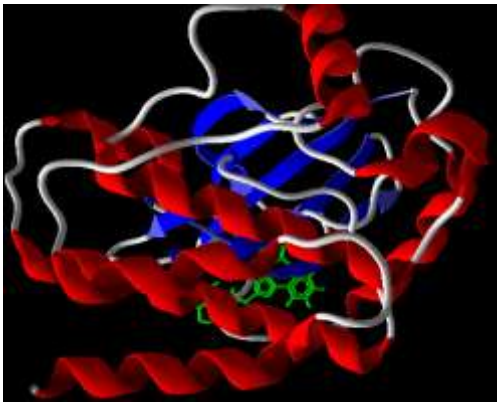
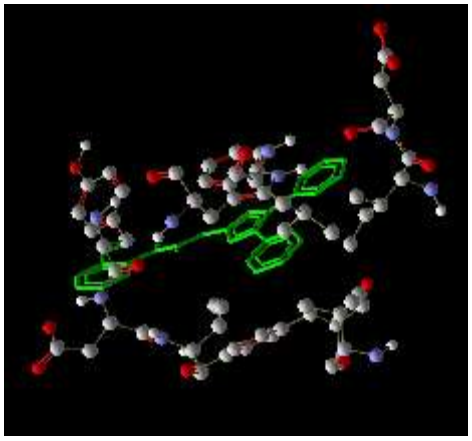
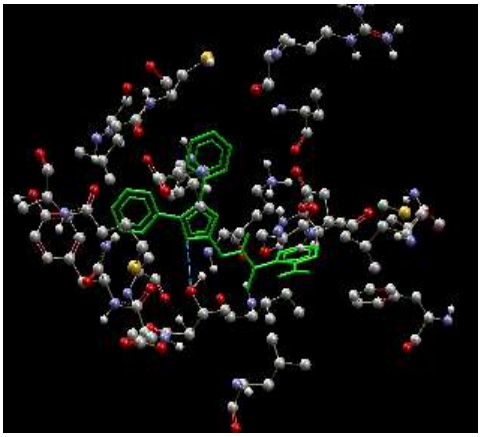


Fig.No.25: Docking view of ligands with active site

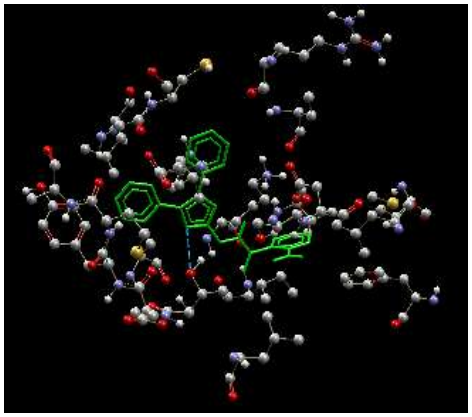
ISA



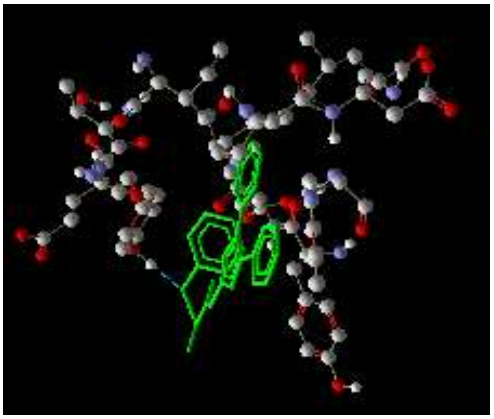
IPABA



IAA



IPAA



IHA

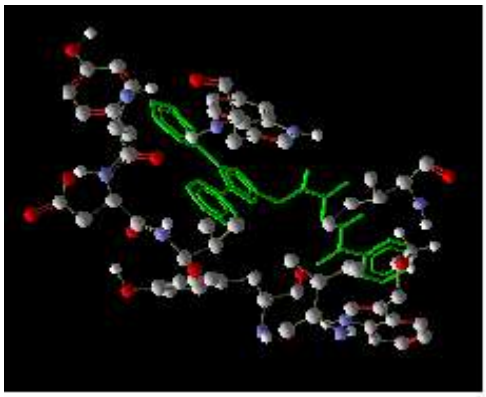
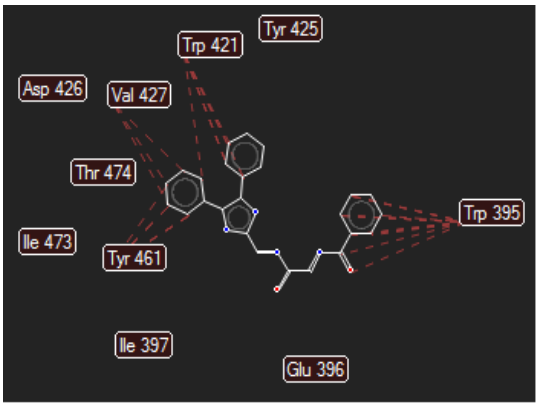
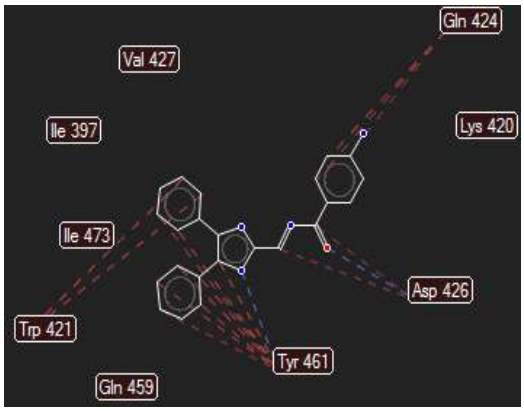


Fig.No.26: Hydrogen bond interaction view of ligand with active site

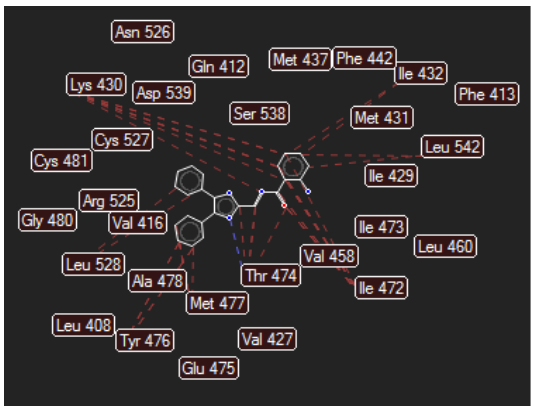
IHA



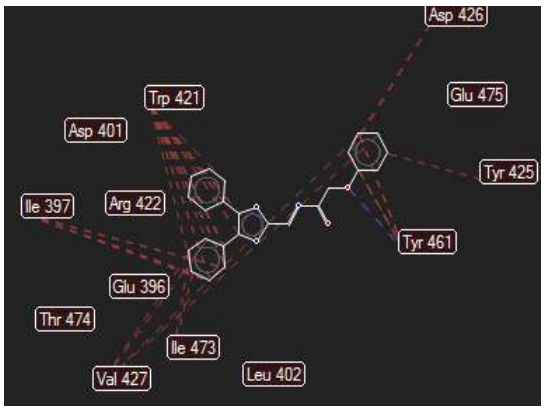
IPABA



IAA



IPAA



ISA

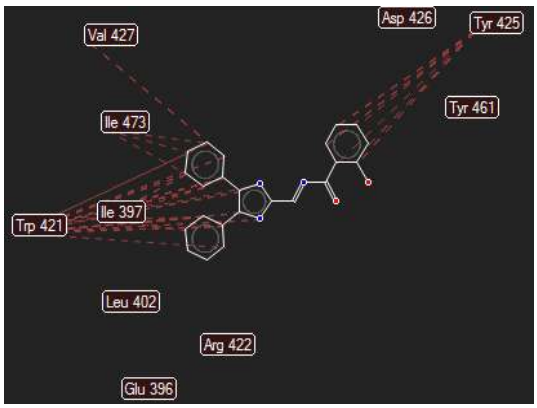


Fig.No.27: Interaction diagram of ligand with active site of target

Table.No.25: Docking Score and interaction of ligands with active site of target using Argus lab 4.1

S.No	Compound code	Enery Score (Kcal/mol)	Type of interaction	Groups involved in the interaction
1	IAA	-11.6513	Hydrogen bond (1) Stearic (7)	Imidazole NH with Thr 474 Lys 430, Ile 432, Leu 542, Thr 474, Met 477, Tyr 476, Leu 528.
2	IPABA	-11.46	Hydrogen bond (2) Stearic (5)	Substitd NH with Tyr 461, Aromatic acid NH with Asp 426 Trp 421, Tyr 461, Gln 424, Asp 426
3	ISA	-11.6045	Stearic (5)	Val 427, Tyr 425, Trp 421, Ile 397, Ile 473.
4	IPAA	-12.7548	Hydrogen bond (1) Stearic (6)	Imidazole NH with Tyr 461 Trp 421, Ile 397, Val 427, Ile 473, Tyr 425, Asp 426.
5	IHA	-12.0101	Stearic (4)	Trp 421, Val 427, Tyr 461, Trp 395.

V. C. EVALUATION STUDIES

i) *IN-VITRO* ANTI-ARTHRITIC ACTIVITY

MATERIALS[56]

Phosphate buffer saline (PH 6.3)

Transfer 8 g of sodium chloride, 0.2 g of potassium chloride, 1.44 g of disodium hydrogen phosphate and 0.24 g of potassium dihydrogen phosphate to a one litre standard flask and dissolve in 800 ml of distilled water. Adjust the pH to 6.3 using 1N Hydrochloric acid and make up the volume to 1000 ml with distilled water.

Bovine serum albumin (5%)

Dissolved 5g of bovine serum albumin in 100 ml water

METHOD

Test solution: 0.5 ml - It consists of 0.45 ml of (5% W/V) Bovine Serum Albumin and 0.05 ml of test solution of various concentrations from10 μg/ml to 1000 μg/ml

Test control solution: 0.5 ml - It contains 0.45 ml of (5% W/V) Bovine Serum Albumin and 0.05 ml of distilled water.

Product control 0.5 ml - It contains 0.45 ml of distilled water and 0.05 ml of test solution

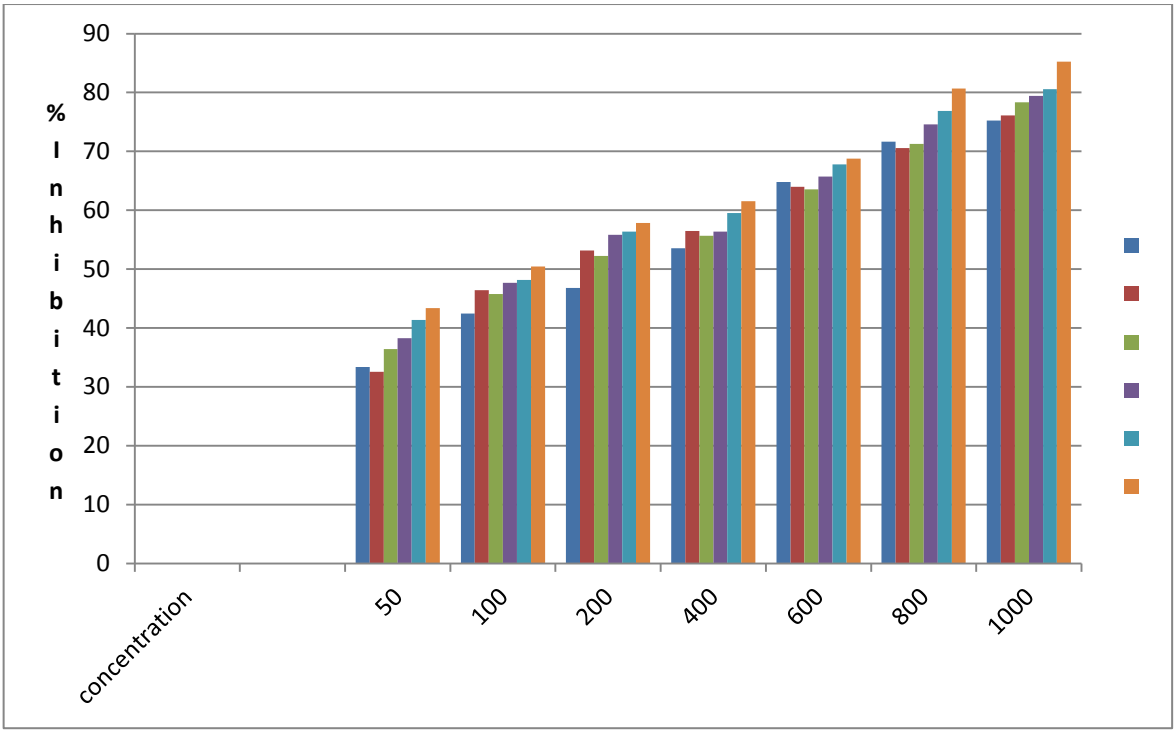
Standard solution 0.5 ml – It contains 0.45 ml of (5% W/V) Bovine Serum Albumin and 0.05 ml of standard drug at different concentrations. The test and standard drug concentration ranging from 50 to 1000 μg/ml (50 μg, 100 μg, 200 μg, 400 μg, 600 μg, 800 μg, and 1000 μg) were taken and the pH was adjusted to 6.3 using 1N Hydrochloric acid. The samples were incubated at 37^{o}C for 20 min and heated at 57^{o}C for 3 min. After cooling 2.5 ml of phosphate buffer was added to all the above solutions. The turbidity was measured at 416 nm using UV-Visible spectrophotometer. The control represents 100% protein denaturation. The percentage inhibition of protein denaturation was calculated using the formula.

$$\% \text{ of inhibition} = \frac{100 - [\text{OD of test solution} - \text{OD of product control}]}{\text{OD of test control}} \times 100$$

RESULTS AND DISCUSSION

Table.No.26: Effect of synthesized compounds and Diclofenac sodium on inhibition of protein denaturation method

S. no	Concentration µg/ml	% of inhibition of protein denaturation					Diclofenac sodium
		IAA	IPAA	IHA	ISA	IPABA	
1	50	33.36±0.02	32.57±0.06	36.43±0.04	38.28±0.04	41.36±0.02	43.36±0.02
2	100	42.44±0.07	46.43±0.05	45.76±0.03	47.67±0.07	48.17±0.09	50.44±0.05
3	200	46.81±0.08	53.18±0.04	52.23±0.02	55.81±0.05	56.36±0.02	57.81±0.04
4	400	53.54±0.05	56.45±0.04	55.65±0.07	56.36±0.02	59.50±0.05	61.54±0.08
5	600	64.79±0.04	63.97±0.05	63.54±0.04	65.73±0.03	67.79±0.05	68.79±0.07
6	800	71.65±0.06	70.55±0.04	71.25±0.06	74.58±0.05	76.89±0.03	80.65±0.04
7	1000	75.26±0.03	76.13±0.03	78.36±0.02	79.43±0.04	80.56±0.06	85.26±0.03



Graph.No.1: Percentage inhibition Vs Concentration

Blue- IAA, Red- IPAA, Green- IHA, Violet- ISA, Sky blue- IPABA, Cyan red- Diclofenac sodium

The *In-Vitro* Anti-arthritic activity has been carried out using most popular inhibition of protein denaturation method. The synthesized compounds showed significant activity at various concentration ranging from 50-1000 µg/ml. From the results the minimum percentage inhibition of protein denaturation of test compounds IAA, IPAA, IHA, ISA, IPABA and standard (Diclofenac sodium) were observed as 33.36 , 32.57, 36.43, 38.28, 41.36, 43.36 at 50 µg/ml and the maximum inhibitory concentration of synthesized compounds were observed as 75.26 %, 76.13 %, 78.36 %, 79.43 %, 80.56 %, 85.26 % at 1000 µg/ml as shown in Table.No.26. At 1000 µg/ml concentration of the synthesized compounds, IPABA was found to effectively inhibit the protein denaturation similar to that of standard drug Diclofenac sodium. Hence, out of all five different synthesized imidazolyl derivatives , compound IPABA was alone considered for further Anti- rheumatoid arthritic evaluation by performing *in-vivo* method.

PHARMACOLOGICAL EVALUATION

a) MATERIALS[64,65]

Animals

Wistar rats (200-300g) either sex were acclimatized for 7 days under standard husbandry conditions, i.e. room temperature 25±10 C, relative humidity 45-55% and light/dark cycle 12/12 hrs. The experimental protocols were approved by the Institutional Animal Ethical Committee (IAEC) of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) **(Regd. No: IAEC/MMC/04/2016)**

Husbandry Practices Caging

Polypropylene rat cages covered with stainless steel grid top were Autoclaved clean rice husk was used as the bedding mate

Water Bottle

Each cage was supplied with a polypropylene water bottle with a stainless steel nozzle.

Housing

Single rat per cage

Room Sanitation

Each day, the floor of the experiment room was swept and all work tops and the floor were mopped with a disinfectant solution.

Animal Identification

Each rat was uniquely numbered on the tail using a tattoo machine. Appropriate labels were attached to the cages indicating the study number, sex, and dose, type of study, cage number and animal number.

b) METHODS

i) Acute oral toxicity (Acute toxic class method in wistar rats)

ii) *In-vivo* anti-rheumatoid arthritis activity.

i) ACUTE ORAL TOXICITY (ACUTE TOXIC CLASS METHOD IN WISTAR RATS)

Acute oral toxicity defines to the adverse effects occurring following oral administration of single dose of substances or multiple doses given within 24 hrs.

The different methods used to evaluate the acute oral toxicity studies are as follows,

i) Fixed dose procedure (OECD Guidelines-420)

ii) Acute toxic class method (OECD Guideline-423)

iii) Ups and Down procedure (OECD Guideline- 425)

Our study was done following acute toxic class method (OECD-423)

OECD Guideline-423

OECD guidelines for the testing of chemicals are periodically reviewed in the light of scientific progress or changing assessment practices. The original guideline 423 was adapted in March 1996 as the second alternative to the conventional acute toxicity test, described in the test guideline 401. Based on the recommendations of several expert meetings, revision was considered timely because international agreement has been reached on harmonized LD_{50} cut-off value for the classification of chemical substances, which differ from the cut-off recommended in the 1996 version of the guideline and testing in one sex (usually female) is now considered sufficient.

A. Acute toxic class method

In the present study the oral toxicity of the synthesized compounds were performed by acute toxic class method. In this methods the toxicity of the synthesized compounds were tested using a stepwise procedure, each step using three rats of a single sex. The various concentration of test drug as per OECD guidelines are as follows, The wistar rats were fasted overnight prior to dosing (food but not water should be withheld). Following the period of fasting the animal should be weighed and the synthesized compounds administered orally at the dose of 2000 mg/kg body weight. Animals were observed individually after dosing atleast during the first 4 hrs and daily thereafter, for a total of 14 days. As no mortality was observed with the above doses, a series of doses 250 and 500 mg/kg body weight were selected for the further pharmacological evaluation. The test procedure with starting doses of 2000 mg/ kg body weight as per OECD- 423 guidelines was shown as follows,

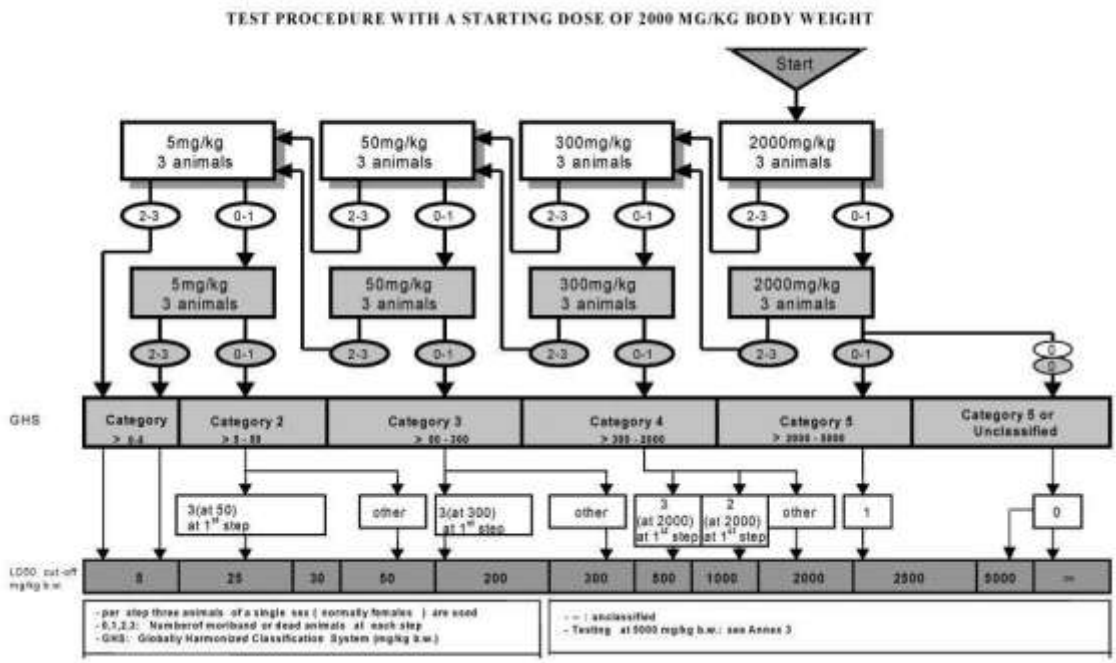


Fig.No.28: Treatment protocol for the acute toxicity study (OECD-423)

ii) *IN-VIVO* ANTI-RHEUMATOID ARTHRITIC ACTIVITY

1. Animal

Protocol of the study was passed by Institutional Ethics Committee of Madras Medical College, Chennai-03. The study was carried out with adult Wistar rats (Male/Female) weighing 100-200 g. Animal were acclimatized to the experimental conditions in cages and kept under a standard environmental condition (22 $\pm$ 3^0C; 12/12 hr light/dark cycle). Rats were allowed to feed and water.

2. Induction of CFA and Drug treatment

Adult wistar rats with an initial body weight of 100-200g were taken, and divided into five groups each containing 6 animals. On zero day, all rats were injected into the sub plantar region of left hind paw with 0.1 ml of Complete Freund's Adjuvant. This consist of *Mycobacterium butyricum* suspended in heavy paraffin oil by through grinding with mortar and pestle to give a concentration of 6 mg/ml. Dosing with test and standard compound was started on zero day and continued for 12 days according to the following schedule: group I: Normal control (Distilled water), Group II: Disease control (suspension of 1% CMC), Group III: Dexamethasone (5 mg/kg, IP. Standard), Group IV: Test compound IPABA (Low Dose) Group V: Test compound IPABA (High dose). From day 13^{th} to 21^{st}, the animals were not dosed with the test compound or the standard. Then the following parameter were measured and tabulated.

Table.No.27: Grouping of animals

Sr. No	Group	No. of Animals	Treatment	Dose
1	Group I Normal Control	6	Normal Saline neither FA treated nor drug treated	----
2	Group II Disease Control	6	Freund's adjuvant (CFA) Distilled water	0.1ml (6mg/ml)
3	Group III Standard Treatment	6	Dexamethasone + CFA	5mg/kg CFA 0.1ml
4	Group IV Cpd-IPABA(low dose)	6	Test compound + CFA	100mg/kg CFA 0.1ml
5	Group V Cpd-IPABA(high dose)	6	Test compound + CFA	200mg/kg CFA 0.1ml

3. **Evaluation of development of Arthritis**

Rats were inspected daily for the onset of arthritis characterized by edema in the paws. The incidence and the severity of arthritis were evaluated using a system of arthritic scoring. Measurement of bi-hind paw volumes every 3 days, when arthritic signs were first visible. Animals were observed for presence or absence of nodules in different organs like ear, fore paw, hind paw, nose and tail. Animal were scored 0 for absence of nodules and 1 for its presence. Score 5 was given as the potential maximum of arthritic score per animal. Hind paw volume was measured using plethysmometer. Paw volumes of both hind limbs were recorded from day of Treatment started to 21st day at three day interval using mercury column plethysmometer.

4. **Rheumatoid Factor**

The latex turbidimetry method was used in the present study using RF turbilatex kit of SPINREACT Company. Calibration was carried out for linear range up to 100 IU/ml. The reading of RF factor of all the groups obtained was compared with the control animals and was expressed as IU/ml RF.

5. **Radiography**

Wistar rats were sacrificed on 21st day of CFA administration and legs were removed and placed on formalin containing plastic bags. This plastic bag was kept at a distance of 90 cm from the X-ray source and Radiographic analysis of arthritic and treated animal hind paw were performed by X-ray machine with a 300-mA exposition for 0.01 s. An investigator blinded for the treatment regimen performed radiograph score. Which were used as a quantal test for bone necrosis. The following radiograph criteria were considered .Radiographs were carefully examined using a stereo microscope and abnormalities were graded as follows:

(i) Periosteaic reaction, 0 - 3 (None, Slight, Moderate, Marked);

(ii) Erosions, 0 - 3 (None, Few, Many Small, Many Large);

(iii) Joint space narrowing, 0 - 3 (None, Minimal, Moderate, Marked);

(iv) Joint space destruction, 0 - 3 (None, Minimal, Extensive, Ankylosis).

(v) Bone destruction was scored on the patella as described previously.

6. Effect on Spleen-Index

At the end of the experiment, after sampled for serum, all mice were sacrificed by ether anesthesia. All the spleens of mice were weighed immediately after dissection. The spleen indexes were calculated by using the following formula:

$$\text{Spleen Index} = \frac{\text{Spleen weight of CFA rat / Body weight of CFA rat}}{\text{Spleen weight of normal rat / Body weight of normal rat}}$$

7. Total leukocyte Count and Neutrophile count

Blood samples were collected by puncturing the retro-orbital plexus into heparanized vials and analysed for total leucocyte counts (TLC) and differential leucocyte counts (DLC)

8. Histological Processing and Assessment of Arthritis Damage

Rats were sacrificed by ether anesthesia. Knee joints were removed and fixed for 4 days in 4% formaldehyde. After decalcification in 5 % formic acid, the specimens were processed for paraffin embedding tissue sections (7 μm thick) and were stained with haematoxylin, eosin or safranin. An experienced pathologist, unaware of the different drug treatments scored the condition of tibiotarsal joints. Histopathological changes were

scored using the following parameters. Infiltration of cells was scored on a scale from 0 to 3, depending on the amount of inflammatory cells in the synovial tissues. Inflammatory cells in the joint cavity were graded on a scale from 0 to 3 and expressed as exudate. A characteristic parameter in arthritis is the progressive loss of articular cartilage. This destruction was separately graded on a scale from 0 to 3, ranging from the appearance of dead chondrocytes (empty lacunae) to complete loss of the articular cartilage. Bone erosion was scored on a scale ranging from 0 to 3, ranging from no abnormalities to complete loss of cortical and trabecular bone of the femoral head. Cartilage and bone destruction by pannus formation was scored ranging from 0, no change; 1- mild change (pannus invasion within cartilage); 2-moderate change (pannus invasion into cartilage/subchondral bone); 3- severe change (pannus invasion into the subchondral bone) and vascularity (0- almost no blood vessels; 1- a few blood vessels; 2-some blood vessels; 3-many blood vessels). Histopathological changes in the knee joints were scored in the femur region on 5 semi-serial sections of the joint, spaced 70 μm apart. Scoring was performed on decoded slides by two observers, as described earlier.

9. **Statistical analysis**

Statistical analysis of difference between groups was evaluated by one-way ANOVA followed by student t test. The values $P < 0.05$ were regarded as significant and the values $P< 0.01$ were considered as highly significant.

RESULTS AND DISCUSSION

A. Oral Acute Toxicity Study OECD-423

Oral acute toxicity of newly synthesized imidazolyl hetero cycles such as IPAA, ISA, IPABA, IHA, IAA were studied and the results were tabulated.

Table.No.28: Observation in Oral Acute Toxicity Study (OECD-423)

S. No	Observation	For 30 mins	4 hrs	24 hrs	48 hrs	7 day	14 day
1	Sedation	Absent	Absent	Absent	Absent	Absent	Absent
2	Excitation	Absent	Absent	Absent	Absent	Absent	Absent
3	Jumping	Normal	Normal	Normal	Normal	Normal	Normal
4	Writhing	Absent	Absent	Absent	Absent	Absent	Absent
5	Scratching	Absent	Absent	Absent	Absent	Absent	Absent
6	Grooming	Absent	Absent	Absent	Absent	Absent	Absent
7	Aggression	Absent	Absent	Absent	Absent	Absent	Absent
8	Ptosis	Absent	Absent	Absent	Absent	Absent	Absent
9	Loss of writhing reflux	Absent	Absent	Absent	Absent	Absent	Absent
10	Lose of pinel reflux	Absent	Absent	Absent	Absent	Absent	Absent
11	Lose of coreneal reflux	Absent	Absent	Absent	Absent	Absent	Absent
12	Excess Salivation	Absent	Absent	Absent	Absent	Absent	Absent
13	Lacrimation	Absent	Absent	Absent	Absent	Absent	Absent
14	Skin & fur	Normal	Normal	Normal	Normal	Normal	Normal
15	Color of eye	Normal	Normal	Normal	Normal	Normal	Normal
16	Tremors	Absent	Absent	Absent	Absent	Absent	Absent
17	Diarrhea	Absent	Absent	Absent	Absent	Absent	Absent
18	Coma	Absent	Absent	Absent	Absent	Absent	Absent
19	Inflammation	Absent	Absent	Absent	Absent	Absent	Absent
20	Urination	Absent	Absent	Absent	Absent	Absent	Absent

In acute toxicity study test, no mortality (sign of toxicity) was observed for all the selected doses during the study. The acute toxicity studies showed that the non-toxic nature of the newly synthesized imidazolyl heterocycles such as IPAA, ISA, IPABA, IHA, IAA was up to the level of 2000 mg/kg body weight selected doses.

B. *In-Vivo* Anti- Arthritic activity

The results were obtained after daily administration of the test doses 100 mg/kg, 200 mg/ kg and standard drug 5mg/ kg in this experimental protocol arthritis revealed that the test compound IPABA exerted the effective anti-arthritic activity.

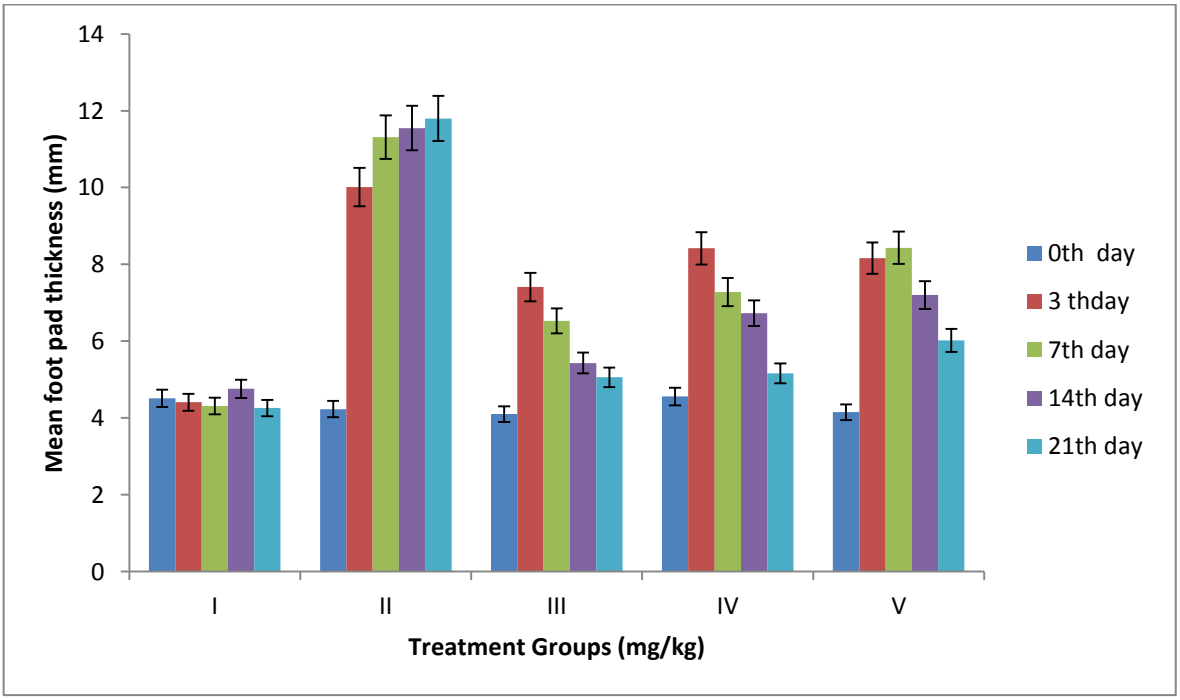
Food pad thickness / Paw volume

The hind paw injected with Complete freund's adjuvant became gradually swollen and reached its peak at 21st day .The result obtained for the two different doses of the test compound and the standard drug (Dexamethasone 5mg/ kg) in the Complete freund's adjuvant induced paw edema test at specific time intervals. When compared with Normal Control rats, the Disease Control rats showed significant increase ($P < 0.001$) in the paw volume after seven days of sub plantar CFA administration. In the primary phase of the arthritis i.e. form day 4 to 7, there was non-significant deceased in the paw volume was observed. Rats treated with test compound (100 and 200 mg/kg) showed significant and dose-dependent attenuation in paw volume from day 12 to 21 onward as compared to Disease Control rats. Rat treated with Dexamethasone (5 mg/kg) significantly decreased ($P < 0.001$) paw volume from day 12 to 21 to as compared to control rats. Rats treated with test compound IPABA (100 and 200 mg/kg) was found to have effective anti-arthritic activity.

Table.No.29: Effect of Foot pad thickness

Treatment	Mean foot pad thickness (mm)				
	0 day	3th day	7th day	14th day	21st day
Group I (NC)	4.51± 0.003	4.41±0.003	4.31±0.003	4.76±0.02	4.26±0.001
Group II (DC)	4.23±0.003***	10.01± 0.003***	11.13± 0.005***	11.55± 0.002***	11.8± 0.002***
Group III (STD)	4.1±0.04***	7.41± 0.04***	6.53± 0.03***	5.53± 0.02***	5.06± 0.02***
Group IV (Cpd-IPABA low dose)	4.56±0.034***	8.42± 0.054***	7.28± 0.02***	6.73± 0.04***	5.16± 0.09***
Group V (Cpd-IPABAhigh dose)	4.15±0.03***	8.16± 0.02***	8.43± 0.02***	7.2± 0.02***	6.02± 0.03***

N= 6, values were expressed as Mean ± SEM, ***p value <0.001 significant, NC- Normal control. DC- Disease control; STD- Standard group; Cpd- Compound IPABA.



Graph.No.2: Foot pad thickness

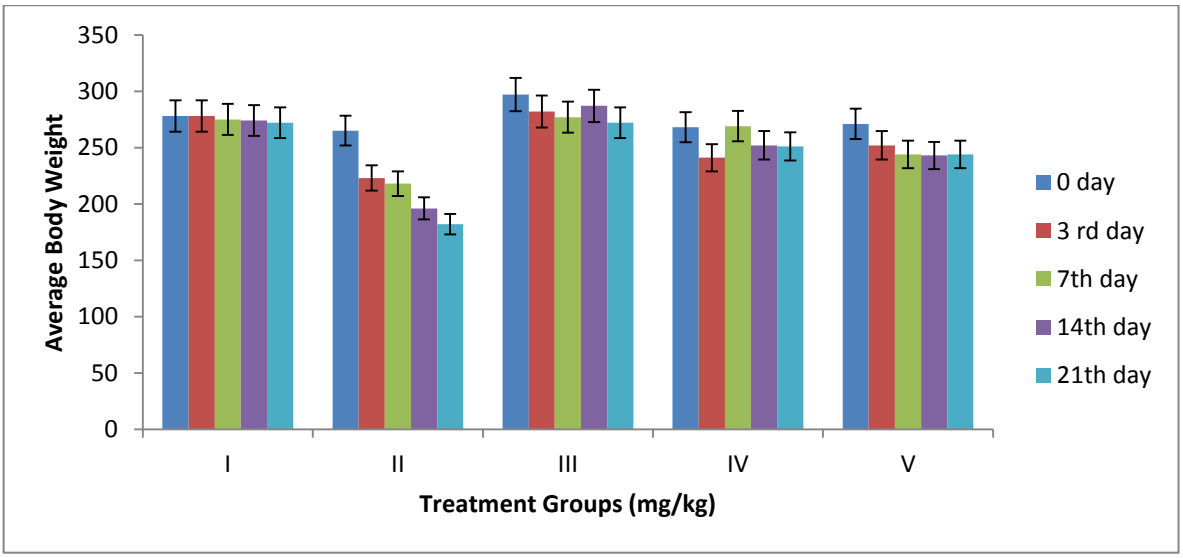
Effect of Average body weight

Changes in body weight was also recorded to evaluate anti- arthritic potential of synthesized compound IPABA. Rats treated with Dexamethasone (5mg/kg) shows significantly increased in body weight (P< 0.001) synthesized compound IPABA 100mg/kg and 200mg/kg also showed significant changes in body weight with value of P<0.001 when compared with disease control.

Table.No.29: Effect of Average body weight

Treatment	Average Body weight				
	0 day	**3rd day**	**7th day**	**14th day**	**21st day**
Group I (NC)	278.66± 0.32	278.32± 0.43	275.50± 0.98	274.60± 0.12	272.93± 0.72
Group II (DC)	265.19± 0.34**	223.16± 0.56**	218.40± 0.34**	196.31± 0.72**	182.41± 0.45**
Group III (STD)	297.18± 0.84**	282.16± 0.96**	277.40± 0.24**	287.31± 0.92**	272.41± 0.32**
Group IV (Cpd low dose)	268.16± 0.764**	241.16± 0.56**	269.40± 0.84**	252.31± 0.42**	251.41± 0.62**
Group V (Cpd high dose)	271.13± 0.24**	252.16± 0.87**	244.40± 0.97**	242.31± 0.32**	244.41± 0.92**

N= 6, values were expressed as Mean ± SEM, ***p value <0.001 significant, NC- Normal control. DC - Disease control; STD - Standard group; Cpd - Compound IPABA.



Graph.No.3: Average body weight

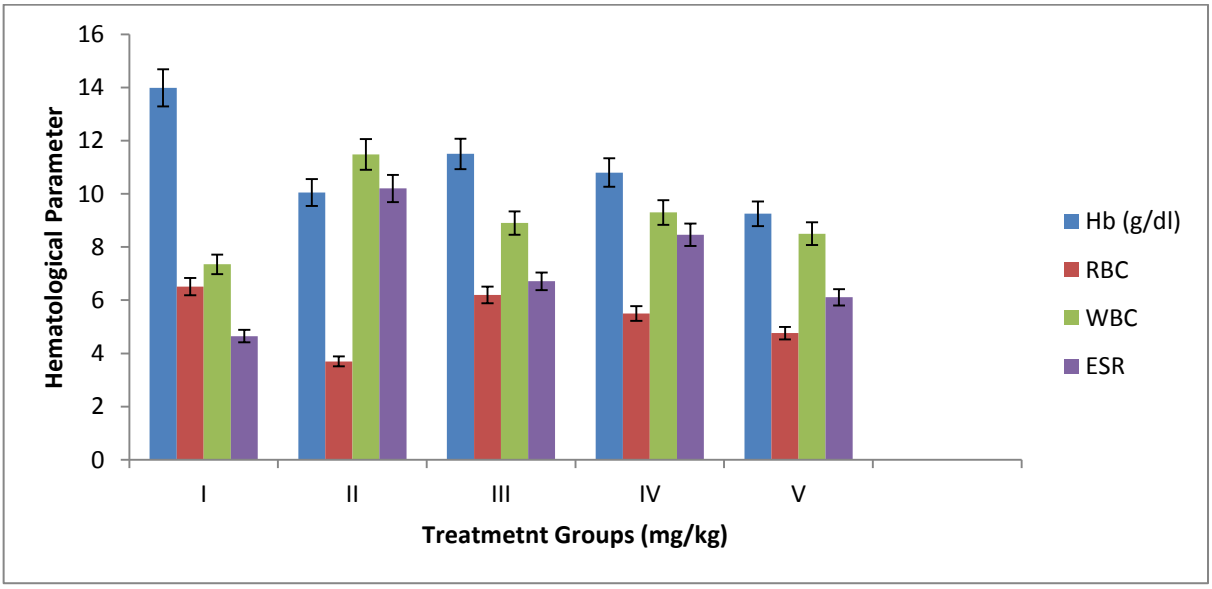
Effect of Hematological Parameter

The changes in hematological parameters in adjuvant induced arthritic rats are shown in Table.No.30. There was a significant ($p < 0.001$) decrease in RBC count and hemoglobin and increase in WBC count and ESR of arthritic rats as compared to control rats. Rats treated with Dexamethasone (5mg/kg) showed changes in the haematological function when compared with disease controlled rats. Rats treated with synthesized compound IPABA 100mg/kg and 200mg/kg also shows significant changes in body weight with value of P<0.001.

Table.No.30: Effect of Hematological Parameter

Treatment	Hb (g/dl)	RBC ($x10^{6}/mm^{3}$)	WBC ($x10^{3}/mm^{3}$)	ESR (mm)
Group I (NC)	13.98 ± 0.040	6.51± 0.030	7.35 ± 0.002	4.65 ± 0.034
Group II (DC)	10.05±0.034***	3.7± 0.025***	11.48 ± 0.030***	10.2 ± 0.025***
Group III (STD)	11.5± 0.025***	6.2 ± 0.025***	8.9 ± 0.025***	6.71±0.030***
Group IV (Cpd low dose)	10.8± 0.025***	5.5 ± 0.025 ***	9.3 ± 0.03***	8.46±0.021***
Group V (Cpd high dose)	9.25± 0.004***	4.76 ± 0.021***	8.5 ± 0.02***	6.11 ±0.03***

N= 6, values were expressed as Mean ± SEM, ***p value <0.001 significant, NC- Normal control. DC - Disease control; STD - Standard group; Cpd - Compound IPABA.



Graph.No.4: Hematological Parameter

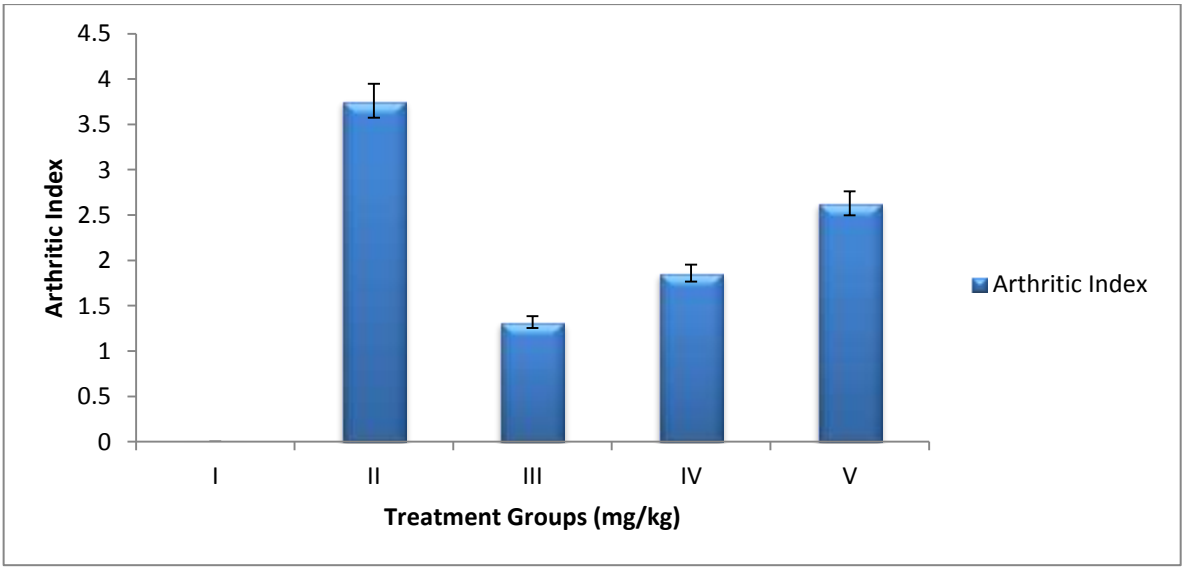
Arthritic Index and Rheumatoid Factor

Sub plantar administration of CFA results in significant increased (P < 0.001) in arthritic score in all arthritic treated rats as compared to control rats. Albino rats treated with synthesized compound IPABA showed significant and dose dependent decreased in arthritic score (P < 0.001) as compared to arthritic diseased rats.

Table.No.31: Effect of Arthritic Index

Treatment	Arthritic Index
Group I (NC)	0.00± 0.000
Group II (DC)	3.76 ± 0.24***
Group III (STD)	1.32 ± 0.25***
Group IV (Cpd low dose)	1.86 ± 0.32***
Group V (Cpd high dose)	2.63 ± 0.18***

N= 6, values were expressed as Mean ± SEM, ***p value <0.001 significant, NC- Normal control. DC - Disease control; STD - Standard group; Cpd - Compound IPABA.



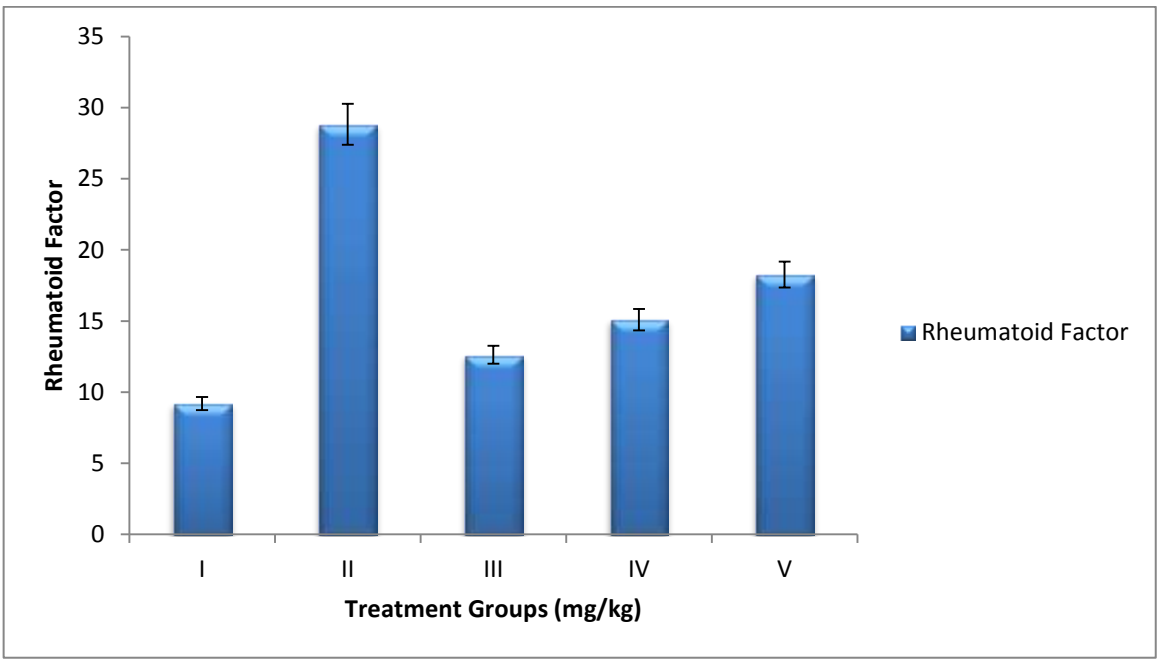
Graph.No.5: Arthritic Index

Arthritic index and Rheumatoid Factor were significantly decreased in treatment with test compound (100, 200 mg/kg) and Dexamethasone (5mg/ kg) treated animal as compared to disease control treatment as shown in Graph.No.5.

Table.No.32: Effect of Rheumatoid Factor

Treatment	Rheumatoid Factor
Group I (NC)	9.20± 0.82
Group II (DC)	28.83 ± 3.39***
Group III (STD)	12.62 ± 2.65***
Group IV (Cpd low dose)	15.1 ± 0.32***
Group V (Cpd high dose)	18.26± 0.18***

N= 6, values were expressed as Mean ± SEM, ***p value <0.001 significant, NC- Normal control. DC - Disease control; STD - Standard group; Cpd - Compound IPABA.



Graph.No.6: Rheumatoid Factor

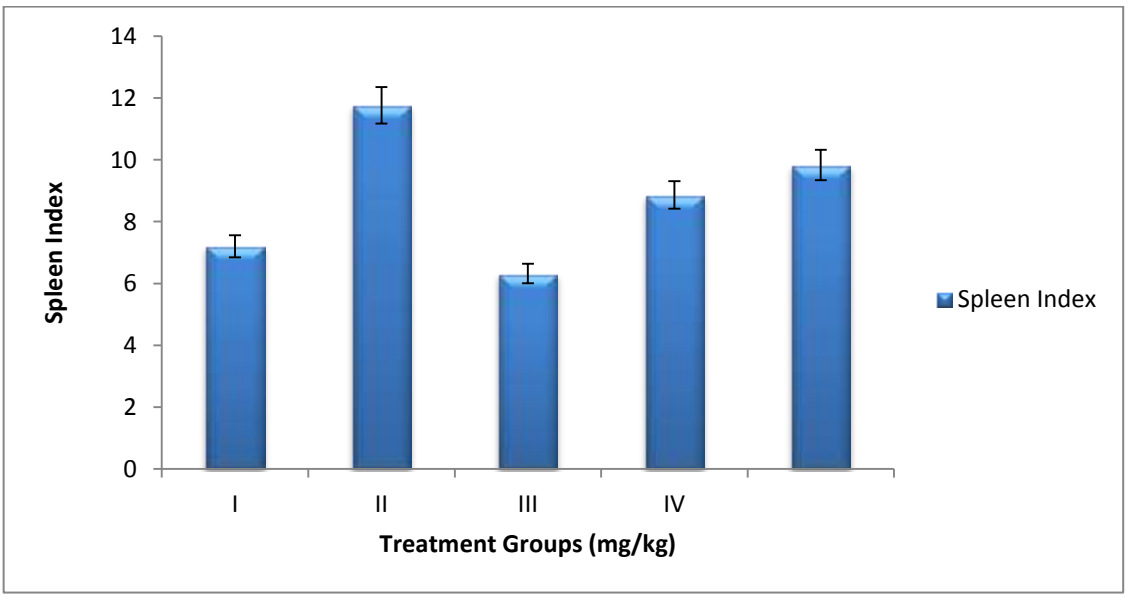
Spleen Index

The spleen provided a readily available source of cells known to be involved in animals. Increased cellularity in the spleen of arthritic rats engendered interest as to the potential for concomitant classical antibody formation synthesized compound IPBA Inhibit splenomegaly, which can enhance inhibitory effect of cells shown in Graph.No.7. Spleen Index were significant increased ($P < 0.001$) in all arthritic treated rats as compared to control rats. The Spleen Index reduction in synthesized compound IPABA (100 & 200 mg/kg) and Dexamethasone (5 mg/kg) treated rats were significantly ($P < 0.001$) lesser than that of arthritic diseased rats.

Fig.No.33: Effect of Spleen Index

Treatment	**Spleen Index**
Group I (NC)	7.2± 0.32
Group II (DC)	11.76 ± 0.24***
Group III (STD)	6.32 ± 0.55***
Group IV (Cpd low dose)	8.86 ± 0.22***
Group V (Cpd high dose)	9.83 ± 0.18***

N= 6, values were expressed as Mean ± SEM, ***p value <0.001 significant, NC- Normal control. DC - Disease control; STD - Standard group; Cpd - Compound IPABA.



Graph.No.7: Spleen Index

Radiography

Bone destruction, which is a common feature of adjuvant arthritis was examined by radiological analysis. Treated rats had developed definite joint space narrowing of the intertarsal joints, diffuse soft tissues swelling that includes the digits, diffuse demineralization of bone, marked periosteal thickening and cystic enlargement of bone and extensive erosions produced narrowing or pseudo widening of all joints space. In contrast, rats treated with synthesized compound IPABA attenuate abnormalities consisted of asymmetric soft tissue swelling and small erosions, periosteal thickening and minimal joint space narrowing areas of the paws and the result were shown below. Despite a similar chemical course of arthritis, disease control rats suffered more pronounced bone destruction than synthesized compound IPABA treated animals as shown on radiograph taken on 21st day in CFA induced arthritis.

Group I (Normal control)

Group II (Disease control)

Group III (Standard control)

Group IV Test (Low dose)

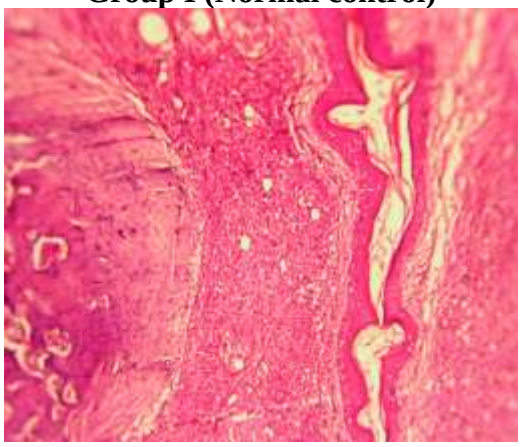
Group IV Test (High dose)

Fig.No.29*: *Effect of synthesized compound IPABA on tibiotarsal joints

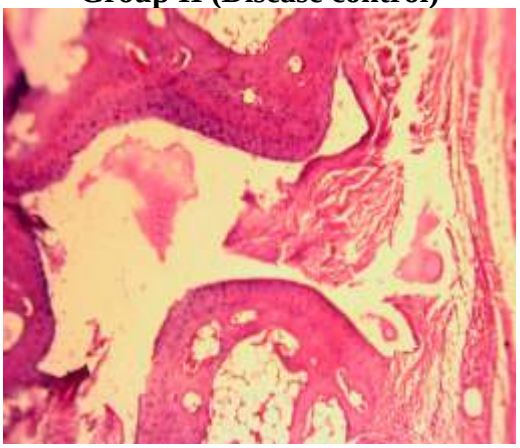
Histopathology

Synthesized compound IPABA treated animals showed more pronounced decrease in bone density, destruction of bony structure, as compare to disease control as shown in Fig.No.30. Abrogation of disease progression by synthesized compound IPABA was further supported by the Histopathologic analysis of the joints from these animals. Rats that had been treated with test compounds at the time CFA immunization showed no histological abnormalities with no evidence of cartilage erosion in their joints in contrast to the disease control rats that displayed completely destroyed joint architecture as shown below

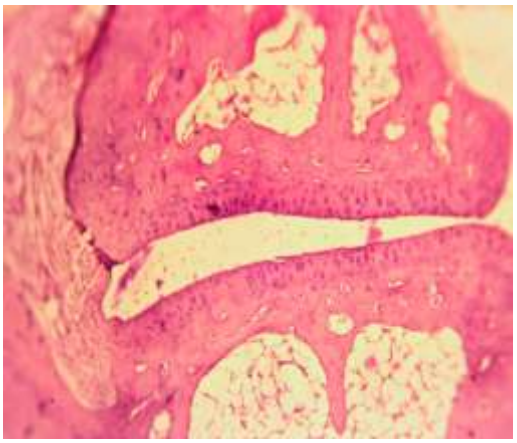
Group I (Normal control)



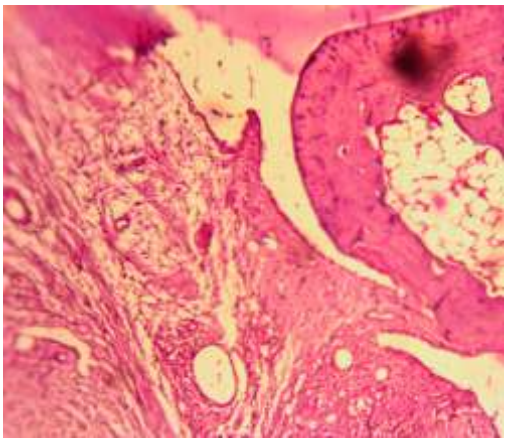
Group II (Disease control)



Group III (Standard control)



Group IV Test (Low dose)



Group IV Test (High dose)

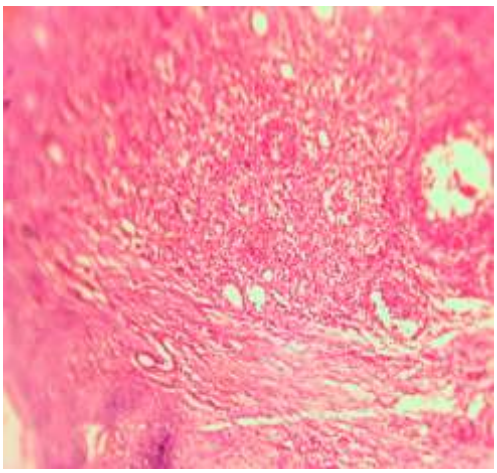


Fig.No.30: Histopathology of joints indicates treatment with synthesized compound IPABA prevent bone erosion

When the synthesized compound IPABA was screened for the *In-vivo* Anti-rheumatoid arthritic activity by adjuvant induced method in which the following parameters were observed and reported as follows

In hematological parameter, the result of P values obtained in one way ANOVA, synthesized compound IPABA 100mg/kg, 200mg/kg was found to decreases the WBC, ESR counts and increases the Hemoglobin, RBC counts with the ***P value < 0.001 and was found to be reduce the rheumatoid factor and compared with that of standard Dexamethasone 5mg/kg.

In spleen index, the result of P values obtained in one way ANOVA, synthesized compound IPABA 100mg/kg, 200mg/kg was found to inhibit the splenomegaly with the ***P value < 0.001 and was found to be reduce the spleen index and also compared with standard Dexamethasone 5mg/kg.

In radiography, the result of P values obtained in one way ANOVA, synthesized compound IPABA 100mg/kg, 200mg/kg was found to increases the joint space and decreases the erosion in joints with the ***P value < 0.001 and proved to be significant when compared with standard Dexamethasone 5mg/kg.

In histopathology, the result of P values obtained in one way ANOVA, synthesized compound IPABA 100mg/kg, 200mg/kg was found to reduces the destruction of bony structure and increased the bone density with the ***P value < 0.001 significantly as that of standard Dexamethasone 5mg/kg.

VII. SUMMARY AND CONCLUSION

Over the last few years, *BTK* has been considered as therapeutic target for selective B-Cell inhibition in the treatment of Rheumatoid Arthritis. Though many *BTK* inhibitors are reported and few are in clinical trials, none are FDA approval. Based on the literature review, all the existing *BTK* inhibitors found to possess mainly five Pharmacophoric features like 1HBAL Hydrogen Bond Acceptor Lipid, 1HBD Hydrogen Bond Donor, 3 HYP Hydrophobic. Hence, a scaffold library has been generated with 33 newly designed ligands which were screened with high docking score against *BTK* using *GLIDE 10.2* and further optimized by Drug likeness properties such as Lipinski rule of five and ADMET properties.

Based on the synthetic feasibility, scaffold containing 4,5 di-phenyl 2-substituted imidazole nucleus like Ligand 6, Ligand 9, Ligand 14, Ligand 13 and Ligand 12 were selected for synthesis. All the selected ligands were chemically synthesized involving Radiswieski reaction and condensation with different aromatic carboxylic acids. Five different synthesized compound such as IPABA, ISA, IAA, IHA, IPAA were prepared and its purity were established by singe spot on TLC plate. The chemical nature of the synthesized compounds were characterized by Melting point determination and different spectral studies such as IR, ^{1}H NMR, ^{13}C NMR and GC-MS analysis. Then the synthesized compounds were subjected to molecular docking studies using both *GLIDE 10.2* and *ARGUSLAB 4.1*.

All the synthesized compounds were subjected to *In-vitro* Anti-Arthritic activity by Protein inhibition assay method at different concentration 50 µg, 100 µg, 200 µg, 400 µg, 600 µg, 800 µg, 1000 µg and also compared with standard Diclofenac sodium. The synthesized derivatives IPABA, ISA, IAA, IHA, IPAA were found to significantly inhibit the protein denaturation. Compounds IPABA was found to effectively inhibit the denaturation of protein at a minimum concentration of 50 µg/ml and maximum concentration of 1000 µg/ml as equipotent as that of Diclofenac sodium.

Oral acute toxicity studies were performed as per OECD guidelines 423 to access the toxicity and also to fix the dose. The LD_{50} value of the test compounds IPABA, ISA, IAA, IHA, IPAA does not found to 2000 mg/kg body weight and also no mortality was observed.

The most potent effective synthesized compound IPABA from the previous *In-Vitro* anti-arthritic screening was selected and subjected to *In-Vivo* anti rheumatoid arthritic activity. When the synthesised compound IPABA was screened at the concentration of 100mg/kg and 200mg/kg for *in-vivo* adjuvant induced arthritic activity, it was found to be equally effective and highly significant (***P value < 0.001) as that of standard drug dexamethasone at 5 mg/kg as proved statistically by one-way ANOVA analysis.

CONCLUSION

Drug design approach as well as clinical studies have revealed that the *tyrosine kinase (Bruton's tyrosine kinase)* have a crucial role in the inhibition of B-cells for treatment of Rheumatoid Arthritis. The present study also provides important structural insights of 4,5 di-phenyl 2 substituted imidazole moieties in designing better *BTK* inhibitor as potent anti- rheumatoid arthritic agents and thus the synthesized compound IPABA (4-amino-*N*-[(4,5-diphenyl-1*H*-imidazol-2-yl)methyl]benzamide) was found to be an effective *BTK* inhibitor for treatment of rheumatoid arthritis.

VIII. REFERENCES

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ANNEXURE

I. PUBLICATION

1. DESIGN AND SYNTHESIS OF SOME NEWER IMIDAZOLYL HETEROCYCLES AS POTENT *BTK* INHIBITORS FOR THE TREATMENT OF RHEUMATOID ARTHRITIS –ACCEPTED.
2. VIRTUAL SCREENING AND PHARMACOLOGICAL EVALUATION OF NEWER *BTK* INHIBITORS AS POTENT ANTI- RHEUMATOID ARTHRITIC ACTIVITY – COMMUNICATED.

II. CPCSEA CERTIFICATE

III. TAMILNADU PHARMACEUTICAL SCIENCES WELFARE TRUST- SCHOLARSHIP AWARDED CIRTIFICATE.

IV. 67^{th} INDIAN PHARMACEUTICAL CONGRESS – PARTICIPATION CERTIFICATE.

V. CME ON PATENCY – PARTICIPATION CERTIFICATE.

VI. WORKSHOP ON DRUG DESIGN CERTIFICATE